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EFFECTS OF CASTRATION AND SUBSEQUENT ANDROGEN ADMINISTRATION UPON MATING BEHAVIOR IN THE MALE HAMSTER (*CRICETUS AURATUS*)[1]

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INTRODUCTION

EXPERIMENTAL investigations and clinical observations have yielded a great deal of evidence concerning the effects of castration upon the sexual capacity of male mammals. The sequalae to adult castration in human beings are ambiguous. Some patients describe pronounced and fairly prompt reduction in erotic responsiveness and capacity for sexual performance. Other castrated men claim that normal libido and potency are retained for several decades (Lange 1934, Daniels and Tauber 1941). This extreme variability might be due to any one of several causes. (1) It is possible that individuals vary endocrinologically, and that extragonadal androgens are in some cases sufficient to support an essentially normal sex life. (2) Claims for continued virility may be false. (3) The patient's expectations and desires may exert a powerful control over his sexual capacities with the result that castration provides a welcome excuse for inactivity or, in contrast, that hormonal deficiencies are compensated by increased "psychic" (cerebral) stimulation.

To weight the various possibilities would indeed be difficult and in any event quite outside the province of this report. More objective

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and reliable evidence on the behavioral consequences of gonadectomy comes from studies of subhuman mammals.

One prepuberally castrated male chimpanzee has been under more or less constant observation for a number of years since the operation. During childhood and adolescence sexual development was normal. Masturbation occurred and sex play was noted. After attaining adult status this prepuberal castrate mated readily and vigorously with receptive females. Save for the absence of ejaculation his behavior was identical with that of an intact and experienced breeder (Clark 1945).

The postcastrational survival of mating behavior in male mammals is not limited to man and the other anthropoids. Evidence dealing with carnivores is scanty but incidental observations made by the senior author suggest that some male cats castrated during adolescence continue to show sexual responses including genital reflexes and copulatory thrusts at least six years after the operation. A study now being conducted by the senior author indicates that there is no marked reduction in the mating performance of male dogs during the first year after adult castration, and subsequent tests of these animals may show that sexual capacity is retained indefinitely.

The consequences of castration in rodents and lagomorphs are quite different. The majority of experimental studies on this subject have dealt with rats, guinea pigs and rabbits and the results are fairly consistent from species to species. Gonadectomy in adulthood is followed by fairly rapid decline in sexual responsiveness. Ejaculation is the first reaction to disappear, and the capacity for erection and intromission is greatly reduced within two or three weeks after operation. By the second postoperative month mating behavior has reached a very low level at which it remains thereafter. This does not, however, represent complete abolition of sexual excitability. In the case of the rat incomplete copulatory responses occur occasionally for many months after testicular loss.

The apparent difference in the effects of castration upon rodents and lagomorphs as contrasted with carnivores and primates raises the interesting question as to whether the dependence of sexual behavior upon androgen is in some way related to phylogenetic status. The experiment to be described here dealt with a rodent species belonging to a genus in which these problems have not been investigated. The golden hamster was chosen as an experimental subject not only because of its systematic position but also because its mating pattern differs in several respects from that of the rat and the guinea pig.

SUBJECTS AND METHODS

The experimental subjects were 19 young adult male hamsters approximately two months of age at the beginning of the observational period. In this species fertile mating begins during the sixth or seventh week after birth (Bond 1945, Reed and Reed 1946). Each male was kept in an individual

cage and all of them lived in a colony room which was darkened from 7:00 A.M. to 7:00 P.M. and well-lighted the remaining 12 hours. This reversal of the light-dark rhythm caused the stimulus females to come into estrus during the day. The animals were fed a diet of lettuce and dog chow supplemented once a week with fresh horse meat. The females used as stimulus animals were the same age as the males and were maintained under similar conditions.

Reed and Reed (1946) have described the copulatory behavior of this species in some detail and our observations agree with theirs in all important particulars. However, instead of adopting the terminology used by these writers we have employed a slightly different one developed in connection with studies of rats, guinea pigs and rabbits. This procedure facilitates interspecies comparisons.

The receptive female flattens her back and stands quietly, allowing the male to mount. Grasping his partner about the flanks the male straightens his hind legs and raises the penis as high as possible, making several rapid, piston-like pelvic thrusts. He may dismount after thrusting several times. Reed and Reed refer to this response as "phase 1" of a copulation. Here it will be termed an abortive or incomplete copulation for reasons to be considered below.

Upon other occasions the male concludes a series of thrusts with a deeper and more vigorous movement in which his pelvis is brought firmly against and slightly beneath that of the female. Then instead of dismounting at once, the male continues to press tightly against the female for 2 or 3 seconds. Reed and Reed named this reaction "phase 2" and stated that it signalled a "successful copulation." We observed hamsters while they mated in a glass-bottomed cage set above a mirror and found that the deep and prolonged final thrust accompanies insertion of the penis, whereas the "phase 1" response never includes intromission. The terms "complete copulation" or "intromission" are therefore used here to indicate the pattern referred to as "phase 2" in Reed and Reed's report.

The earlier workers were able to determine with "reasonable accuracy" the occurrence of emission or ejaculation by observing the temporal spacing of successive mounting reactions and the intervening rest periods. Our animals did not behave in the manner described by Reed and Reed and we were forced to conclude that any relation between ejaculation and observable behavior was too variable to be relied upon.

Before each day's tests receptive females were chosen on the basis of their response to a sexually active "indicator male." The experimental male was put in a small glass aquarium and left there for at least five minutes in order that he might become adapted to the environmental situation. Preliminary observations showed that even when a receptive female was present a male was likely to spend the first few minutes investigating the environment. Males that were allowed to carry out this exploration before the test began tended to initiate mating responses promptly as soon as the female was presented.

At the end of the adaptation period a receptive female was introduced and a timer was started. If no intromissions were achieved within ten minutes after the female's entrance the test was scored as negative. If the male did effect intromission in 10 minutes or less observations were continued for 15

minutes from the time of the first intromission. As the test proceeded the frequency of intromissions and abortive copulations was recorded and notes on other aspects of the males' performance were made.

Each male was given 10 sex tests before castration, the tests being spaced at intervals ranging from 3 to 21 days. Analysis of the results indicated that within these limits the length of the rest period between tests has no effect upon sexual behavior. The experimental population was divided into five groups equated as nearly as possible in terms of average frequency of intro-

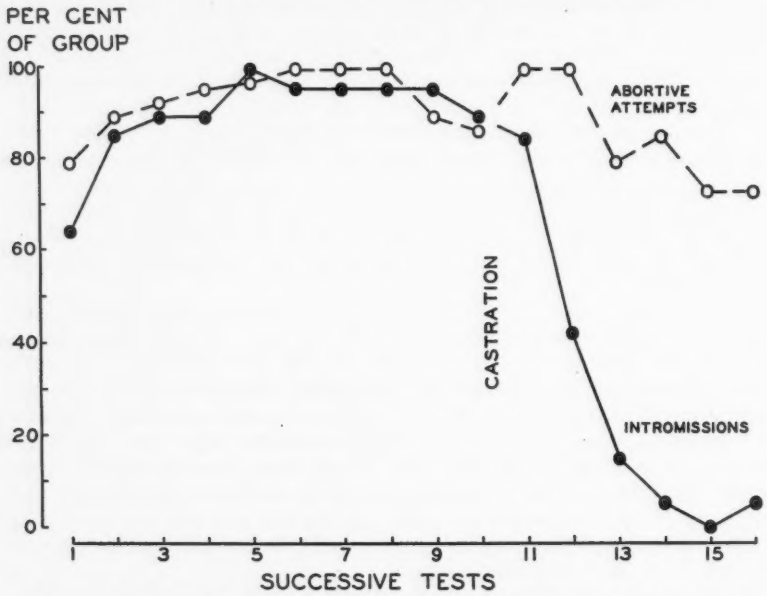

FIG. 1. Percentage of the group achieving at least one intromission in each test before and after castration (solid line) and percentage executing at least one mount without intromission (broken line).

missions per preoperative test. One group was first tested on the third day after castration, another on the fourth day and one each of the remaining castrate groups received their initial postoperative tests on the fifth, sixth and seventh days after gonadectomy. Thereafter all animals were tested once a week for six weeks. Analysis of results failed to show any reliable change in sexual performance for intervals of less than a week and therefore in preparing this report the postoperative records of the five subgroups have been combined.

Three months after castration, and thus six weeks after the sixth post-castrational mating test, one brief test was given and daily injections of testosterone propionate were begun.[2] One group of castrates received 1 microgram (.001 mg) of androgen daily for a period of two weeks. Since this

[2] The testosterone propionate employed in these experiments was Oreton generously supplied by Dr. Edward Henderson of Schering Corporation, Bloomfield, N. J.

dosage had no apparent effect upon sexual behavior the concentration was raised to 100 micrograms per 24 hours and sex tests were continued. A second group of castrates was treated with 75 micrograms per day from the beginning of the injection period.

At the beginning of the third week of the injection period daily sex tests were conducted and these continued until the complete copulatory response (intromission) had reappeared. If, during his first positive test, a particular male performed as well as he had before operation his tests were considered completed and he was dropped from the experiment. If, during the test in which intromission first reappeared it was performed only a few times, the male was allowed a week's rest, daily injections were continued and additional sex tests were conducted at the rate of one per week. The weekly tests were continued until the male had been restored to preoperative potency or until it became necessary to terminate the experiment.

RESULTS

Number of males achieving intromission

The solid line in figure 1 shows the proportion of the group achieving one or more intromissions in tests conducted before and after castration. In the course of the first few tests there was some increase in the number of males responding, but during each of the last 5 preoperative tests from 90 to 100 per cent of the group displayed this response at least once. Within two weeks after castration the number of animals achieving intromission was greatly reduced. As the postoperative interval lengthened the group performance continued to decline until during the fifth week after castration none of the 19 males effected intromission.

Power of intromission was not completely lost, however, for one week later two males achieved intromission once or twice; and in a brief test given three months after castration this reaction was shown a limited number of times by two other individuals. It is possible that some slight capacity for intromission may survive indefinitely despite the absence of testicular hormone.

Frequency of intromissions per male per test

The actual frequency with which intromission is achieved by normal male hamsters is subject to considerable individual variation. Before operation our animals showed a range of 1 to 85 intromissions in separate tests and the average frequency per preoperative test ranged from 18 to 73 for different males. These averages are based upon only those tests in which intromission occurred at least once. Despite the wide variability of individual performance, the intromission score in successive tests proved to be reasonably consistent for any given male. The odd-even reliability was $+.89 \pm .03$.

During the first four days after removal of the testes there was no significant reduction in the frequency of intromission, but with longer postoperative intervals this response decreased markedly. The solid

line in figure 2 shows the average frequency of intromission during those tests in which this reaction appeared at least once. As seen in figure 1 the number of males continuing to achieve intromission was rapidly reduced during the first few postoperative weeks; and figure 2 reveals that even when this response survived it occurred less and less frequently in successive postoperative tests. Before operation the average intromission frequency ranged from 34 to 52 in different tests, but those males which succeeded in effecting intromission during the test given one month after castration did so an average of only 13

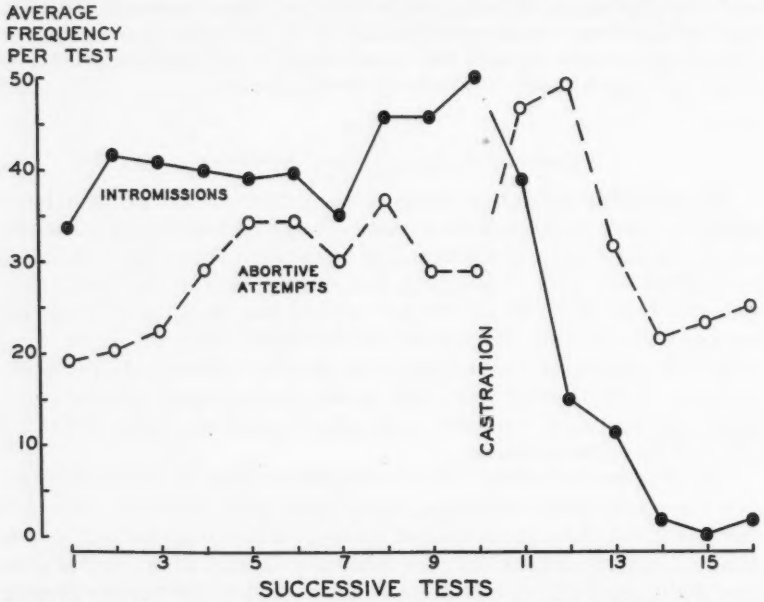

FIG. 2. Average frequency of intromissions per male per test before and after castration (solid line) and average frequency of mounts without intromission (broken line).

times. Including all postoperative tests in which intromission occurred, the average frequency was found to be 32.2 responses per test as compared with a preoperative average of 43.1. This difference is highly significant according to Student's t test ($P = .01$).

It is not clear why some castrates retained copulatory activity longer than others but there is some suggestion of a positive relationship between pre- and postoperative sexual vigor. The experimental subjects were ranked according to the greatest number of intromissions achieved in any one test after operation, and they were also ranked according to average intromission frequency in preoperative tests. The coefficient of correlation between these two rankings was $+.71 \pm .08$.

With respect to the effects of castration upon the intromission response three generalizations are indicated. (1) The number of males achieving intromission declined rapidly in successive weeks after the operation and after approximately one month less than 10 per cent of the group responded in any single test. (2) Those males that did exhibit intromission postoperatively were able to do so only a limited number of times. The actual frequency of this reaction dropped off rapidly and by the end of the first postoperative month the few individuals capable of responding effected only two or three intromissions during a 15 minute period with the receptive female. (3) Our results suggest that some slight capacity for intromission survives in at least some individuals as long as three months after removal of the testes.

Number of males showing abortive attempts

The broken line in figure 1 shows the per cent of the experimental group exhibiting at least one abortive or incomplete copulatory attempt in each of the tests. The proportion of the group responding increased during the first few tests and ranged between 90 and 100 per cent for the last seven preoperative tests. Following castration this measure of sexual activity showed little change for the first two weeks, and even six weeks after operation three-quarters of the males made abortive attempts to copulate. During a brief test given three months after castration 14 of the 19 males made one or more of these responses.

Differences between the two graphs in figure 1 suggest that the tendency to mate survives longer after castration than does the ability to achieve intromission. It seems likely that hormonal deficiency leads to failure of erection despite the fact that the remaining sexual excitability is sufficient to call forth numerous copulatory attempts.

Frequency of attempts per male per test

Before operation the experimental males frequently mounted the female without achieving intromission. The average scores per test ranged from 12.1 to 41.5 for different individuals. Test-to-test consistency was fairly high and the odd-even reliability coefficient was $+.76 \pm .07$. The broken line in figure 2 shows the average frequency of abortive copulations in each successive test. For the first two weeks after castration the number of incomplete contacts increased well above preoperative averages. Thereafter the frequency of this response returned to preoperative levels and showed no further decline during the test period. It seems clear that castration did not eliminate mating tendencies although it did greatly reduce the probability of completed copulations with intromission.

Initial latencies

The initial latency was the number of seconds elapsing between the introduction of the receptive female and the male's first intromission. In preoperative tests this function showed a low negative correlation with intromission frequency ($-.43 \pm .13$). In other words, males that were slow to achieve their first intromission tended to effect fewer complete copulations in a time-limited test than did those individuals that initiated sexual contact with less delay.

In order to normalize their distribution the latency scores were translated into log values and the result is shown in figure 3. Little

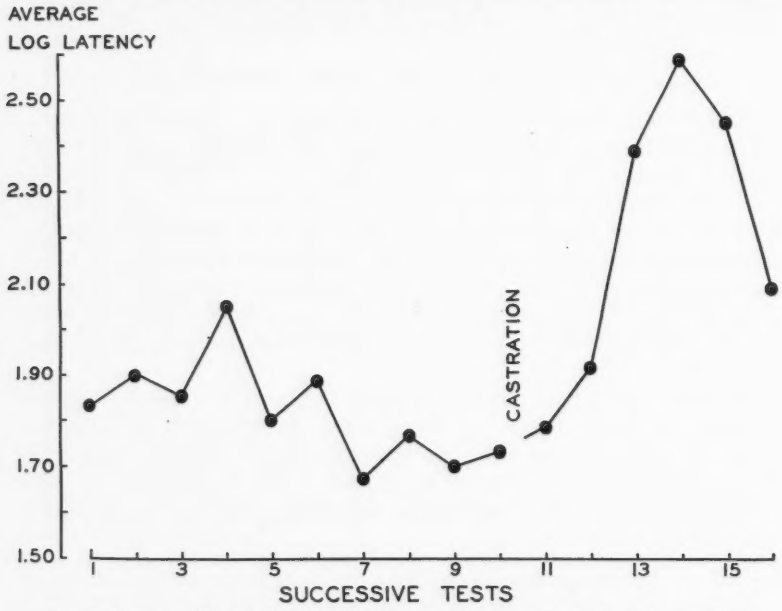

FIG. 3. Average delay before the first intromission in pre- and post-operative tests expressed in terms of log seconds.

change occurred until the third postoperative week, but at this time the delays grew appreciably longer than they had been prior to testicular removal. During the last three tests in the postoperative period so few males achieved intromission that group averages were highly variable. For this reason the apparent decrease in latency on tests 15 and 16 is of doubtful significance but there is little question that the untreated castrate tends to delay for more than the normal period before achieving complete sexual contact with a receptive female.

Effects of androgen treatment

It has been stated that daily injections of testosterone propionate were begun 90 days after castration. Those males receiving 1 micro-

gram per day showed no increase in sexual activity after two weeks of such treatment and accordingly the dosage was increased to 100 micrograms. The remaining animals received 75 micrograms per day from the start of the injection period. Both of these larger doses proved effective in reviving copulatory ability.

Unfortunately it was impossible to continue the experiment until all males had been restored to a normal level of sexuality. However, in five cases this result was achieved after a total of 975 to 1500 micrograms had been administered. In each of these five cases the copulatory performance as measured by intromission frequency equalled or exceeded the average preoperative performance.

Several other individuals displayed increased sexual ability under the influence of testosterone propionate but our results do not permit us to state whether continued treatment would eventually have restored fully normal mating behavior in all cases. The important point which can be made is that in the castrated hamster as in the rat, guinea pig and rabbit exogenous male hormone facilitates the maintenance or restoration of capacity for sexual arousal and for copulatory performance.

DISCUSSION

Although they showed some decrease in weight after castration, our animals appeared to be in excellent health and regained most of the lost weight before the end of the experiment. We do not believe that the weight loss exerted any appreciable effect upon sexual performance. Any effect of operative shock should have been most evident in tests conducted shortly after castration, but in these tests the hamsters showed more intromissions than were executed in subsequent observation periods. Furthermore, as body weight gradually increased there was no parallel increase in sexual activity which is what might have been anticipated if postoperative changes were due primarily to general ill health. Finally, when the administration of testosterone propionate restored mating behavior it did not cause any appreciable change in body weight. It seems safe to conclude that postcastrational changes in sexual performance were due to a deficiency in gonadal hormone and not to some more general systemic effect of the operation.

The effects of castration upon sexual behavior in the hamster and the rat are closely comparable in some respects. If the behavioral change is expressed in terms of the proportion of the group achieving intromission in successive postoperative tests, the performance curves for the two species are practically identical. When intromission does occur after castration the average frequency with which it is achieved in a time-limited test is progressively reduced as the postoperative interval increases. The rate of reduction in this function appears to be about the same in hamsters and rats. Furthermore both species seem to retain some slight capacity for intromission for at least several

months after castration. Finally, sexual behavior can be revived in castrated rats and hamsters by administration of testosterone propionate.

Certain inter-species differences appear to be significant. In the case of the castrated rat abortive attempts to copulate survive somewhat longer than does the capacity for intromission, but these incomplete mating reactions are greatly reduced within a month after gonadectomy. In the hamster, in contrast, this type of sexual response is less easily affected by castration. During the first postoperative month there is little or no decrease in the frequency with which copulation is attempted, and three months after gonadectomy the behavior was still present in 75 per cent of the males examined in the present study. The fact that abortive copulations continue long after the power of intromission has almost disappeared gives a clue to the degree to which these two phases of sexual activity depend upon testicular hormone.

It has previously been shown that in the male rat the ability to achieve intromission may be retained under the influence of hormonal concentrations too low to maintain the power of ejaculation (Beach and Holz-Tucker 1949). In this species, however, it has proven difficult to maintain or restore the incomplete copulatory response without at the same time evoking complete copulation with intromission. In the hamster this separation is readily achieved.

SUMMARY

Nineteen male hamsters were observed in a series of 10 mating tests with receptive females and then castrated. Sex tests were continued during the first six weeks after the operation. Three months postoperatively daily injections of testosterone propionate were initiated and mating tests were resumed.

Castration was followed within two weeks by a marked decrease in the number of males achieving intromission. Within one month this response had been so greatly reduced that it was shown by only one or two individuals in any given testing period. It did not, however, completely disappear from the repertoire of all animals in the space of three months after castration.

The average frequency of intromissions per male per test was definitely reduced within two weeks after operation. Further decreases followed with the result that when this reaction did appear it was executed only two or three times in 15 minutes as contrasted with 40 or more times during similar tests before operation.

Abortive copulatory attempts which did not include intromission occurred in the behavior of all males both before and after castration. The number of tests in which this response appeared was only slightly decreased by castration, and the frequency per male per test was as

high during the six weeks of postoperative testing as it had been before operation.

The amount of time elapsing before a male achieved the first intromission of a given test tended to increase in castrated animals during the first three weeks after gonadectomy. In later tests intromission occurred so infrequently that group averages became highly variable.

Normal mating behavior was revived in 5 of the 19 castrates by androgen treatment which began three months after operation. In other individuals an improvement in sexual performance was noted but injections were terminated before full normality had been restored.

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ANAEROBIC GLYCOLYSIS IN RAT OVARIAN TISSUES DURING PREGNANCY AND LACTATION[1]

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SEVERAL reports have been made from this laboratory on the changes in the activities of certain enzymes in ovarian tissues of the rat during different phases of the reproductive cycle. The adenosine triphosphatase activity of corpora lutea and the remaining ovarian tissues has been studied (Biddulph, Meyer and McShan, 1946a, 1946b) as well as the succinic dehydrogenase (Meyer, McShan and Erway, 1945; Meyer, Soukup, McShan and Biddulph, 1947), malic dehydrogenase (McShan, Erway and Meyer, 1948) and the acid and alkaline phosphatases (Stafford, McShan and Meyer, 1947).

Recent advances in the manometric determination of anaerobic glycolysis of tissue homogenates (Racker and Krimsky, 1945; Utter, Reiner and Wood, 1945; and Novikoff, Potter and LePage, 1948) have made it feasible to determine the anaerobic glycolysis of ovarian tissues during pregnancy and lactation in the rat. The method used was essentially that of the latter workers.

MATERIALS AND METHODS

The rats used in this study were of the Holtzman strain, approximately four months of age. The females were bred and the day following insemination was considered to be the first day of pregnancy. The day following parturition was designated the first day of lactation.

The number of young was limited to 6 to 9 until the 5th day of lactation and 6 young thereafter. The animals were killed by decapitation on days 4, 7, 11, 15 and 20 of pregnancy and of lactation, and on the second day of diestrus.

The ovaries were removed immediately and the corpora were dissected out as rapidly as possible on moist hard surface filter paper. During lactation the corpora of pregnancy are distinguished from those of lactation since the latter are lighter in color and have more prominent ovulatory papillae; the corpora of lactation are also larger after day 7 of lactation than those of

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pregnancy. The corpora of each type were counted, weighed collectively and placed in a homogenizing tube containing 0.2 ml. of glass distilled water and which was surrounded by an ice bath. The ovarian residues remaining after dissection of the corpora lutea were weighed and placed in a similar homogenizing tube. The tubes containing the tissues were kept in an ice bath during homogenization and until the homogenates were added to the Warburg flasks. Sufficient cold glass distilled water was added during homogenization to make a 5 per cent homogenate and 0.3 ml. of each kind of homogenate was used in each Warburg flask.

The tissue from two rats was pooled for each determination of diestrous corpora. Sufficient tissue was obtained from a single rat for each determination of the tissues of pregnancy and lactation. If enough tissue was available, duplicate flasks were used. When tissue from a single animal was insufficient for duplicate flasks, only one flask was used. The activity of liver homogenate was simultaneously measured using duplicate flasks so as to show that the purity of the reagents and manipulative procedures were satisfactory.

The method of Novikoff, Potter and LePage (1948) was used for determining the anaerobic glycolysis activity of the ovarian tissues. Slight modifications of this method were found necessary for optimal activity of the tissues used in the present study. Warburg flasks with side arms were used. The flasks contained the following components added in the order cited: Glass distilled water to make a final volume of 3.0 ml., 0.3 ml. of 0.1 M K_2HPO_4-KH_2PO_4 (pH 7.6), 0.3 ml. of 0.5 M $KHCO_3$, 0.1 ml. of 0.01 M potassium adenosinetriphosphate (ATP), 0.15 ml. of 0.2 M nicotinamide, 0.2 ml. of $\frac{1}{2}$ per cent potassium diphosphopyridine nucleotide (DPN) (added to the side arm) 0.6 ml. of 0.04 M potassium hexosediphosphate, 0.3 ml. of 0.28 M glucose, 0.1 ml. of 0.1 M $MgCl_2$, 0.3 ml. of 0.01 M potassium glutathione (GSH) (when used), 0.2 ml. of 0.015 M potassium pyruvate (freshly prepared from 1.0 M pyruvic acid) and 0.3 ml. of 5 per cent tissue homogenate (equivalent to 15 mg. of fresh tissue). All components except the last three were added before the animal was killed and the flasks were kept at refrigerator temperature until ready for use.

The DPN was prepared according to the LePage (1947) modification of the Williamson and Green (1940) method. Assay by means of the malic dehydrogenase system showed the product to be at least 50 per cent pure. The ATP was prepared as the barium salt according to the method of Needham (1942). The inorganic phosphorus liberated by boiling ATP in 1 N HCl for 7 minutes showed this product to be 90 per cent pure. Hexosediphosphate was prepared from the barium salt manufactured by Schwarz Laboratories, Inc. Pyruvic acid obtained from the Eastman Kodak Co. was redistilled under vacuum, diluted to approximately 1.0 M and accurately titrated, then diluted and neutralized with K_2CO_3 shortly before use.

After the addition of the homogenate, the flasks were attached to the manometers and gassed from a manifold with 95% N_2:5% CO_2 for 10 minutes. They were then placed in the bath and equilibrated for 5 minutes. The DPN was then tipped in from the side arms. After 5 minutes the zero reading was taken and the measurement of CO_2 evolution begun. Readings were taken at 10 minute intervals for the next 80 minutes. It was found that the rate of reaction was consistently linear with respect to time from minutes

20 to 80; for this reason the $Q_{CO_2}^{N_2}$ values (microliters of CO_2 given off under anaerobic conditions per mg. of dry tissue per hour) were based on this period. The dry weights used for the calculations of this Q value were those obtained by Meyer et al. (1947) (Diestrous corpora, 24.4%, corpora of pregnancy, 22.8%, corpora of lactation, 21.4% and ovarian residue, 22.4%).

RESULTS AND DISCUSSION

The anaerobic glycolytic activity of the ovarian residue throughout pregnancy and lactation and of the corpora of diestrus, pregnancy and lactation, together with their weights are recorded in Table 1.

TABLE 1. GLYCOLYTIC ACTIVITY OF LUTEIN TISSUE AND OVARIAN RESIDUE AND WEIGHT OF CORPUS LUTEUM DURING PREGNANCY AND LACTATION

Stage	No. of expts.	Glycolytic activity ($Q_{CO_2}^{N_2}$) Corpora of:		Ovarian residue	Weight (mg.) Corpora of:	
		Pregnancy	Lactation		Pregnancy	Lactation
Diestrus	6	10.2 ±2.54*		—	1.22 ±0.14**	
Pregnancy days						
4	4	16.4 ±1.84		10.2 ±1.07	1.60 ±0.17	
7	4	16.8 ±0.99		9.6 ±4.17	1.78 ±0.15	
11	6	17.4 ±1.79		9.3 ±3.29	2.50 ±0.62	
15	6	16.0 ±1.43		7.5 ±1.85	4.23 ±0.28	
20	4	16.0 ±1.82		8.8 ±1.91	4.40 ±0.15	
Lactation days						
4	5	12.8 ±0.70	16.6 ±1.69	5.2 ±0.68	2.65 ±0.16	1.83 ±0.19
7	2	14.4	18.2	6.1	1.84	2.18
11	4	14.0 ±2.33	18.1 ±1.18	5.8 ±3.67	1.42 ±0.12	2.11 ±0.11
15	4	—	17.2 ±0.71	5.5 ±1.68	—	2.56 ±0.16
20	4	—	17.5 ±0.94	7.2 ±4.36	—	2.38 ±0.33

* Standard deviation.

** This figure is not a true standard deviation. The desired corpora from each rat were weighed collectively and the average corpora weight calculated from the total weight. This figure is the standard deviation from the average weight determined in each collective weighing.

The glycolytic activity of the corpora together with their weights are graphically presented in Figure 1. The weights of the corpora of pregnancy and of lactation are essentially the same as those obtained by Biddulph, Meyer and McShan (1946a).

There is a marked increase in the glycolytic activity of the corpora from the second day of diestrus to the 4th day of gestation. From day 4 to 11 of pregnancy there is no significant change in activity. After day 11 there is a slight decline in activity of the corpora of pregnancy until day 20, and a more rapid decrease to the 4th day of lactation. Statistical analysis of the data on hand shows only two significant changes in the glycolytic activity of lutein tissue. The first of these is between the second day of diestrus and day 4 of preg-

nancy while the second such change occurs in the corpora of pregnancy between day 20 of pregnancy and day 4 of lactation.

The enzymatic activity of the diestrous corpora was determined in order that it might serve as a base line with which to compare the enzymatic activity of lutein tissue during pregnancy and lactation. It can not be assumed, however, that the corpora of diestrus are functionally similar to those of early pregnancy, since it is believed by some workers that the corpora of diestrus are non-functional, or, at most, under low-grade stimulation (Everett, 1945), while those of early pregnancy are highly functional and under the direct stimula-

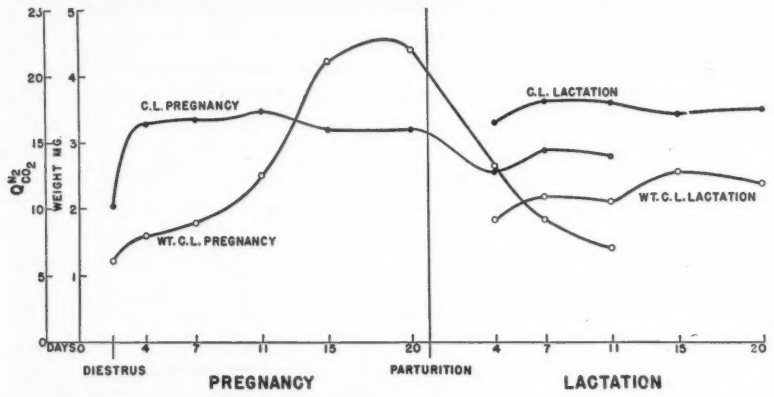

FIG. 1. The anaerobic glycolysis and weight of the corpora lutea of pregnancy during pregnancy and lactation, and of the corpora of post-partum ovulation during lactation.

tion of the pituitary (Everett, 1947). This difference in the functional state could well be reflected in the differences in the glycolytic activity of the two types of corpora.

There is no appreciable change in the activity of the corpora of post-partum ovulation except that it is higher than the activity of the corpora of pregnancy. The glycolytic activity of the ovarian residue shows no noteworthy change other than a gradual decrease during pregnancy and lactation.

In order to check the likelihood that the reconstituted system used in these determinations might be limiting the enzymatic activity, homogenates of other rat tissues such as kidney, brain and liver were tested under identical conditions. The glycolytic activity of these tissues was substantially higher than that of luteal tissue. The activity of liver in terms of $Q^{N_2}_{CO_2}$, was as great as 42. From these data it is evident that the system used will measure greater enzyme activity than that present in the ovarian tissues.

It has been found in this laboratory (unpublished data) that glu-

tathione will activate the anaerobic glycolysis of liver and brain homogenates as measured by lactic acid production and CO_2 evolution. The mechanism of this activation is, at present, not clear but is probably concerned with the protection of the -SH enzymes of the glycolytic system (see Barron and Singer, 1943; and Cori, Slein and Cori, 1948). It should be noted, however, that LePage (personal communication) found that GSH had no effect on the glycolytic rate although his procedure did not differ from ours in any discernible manner.

Whatever the final outcome of this activation study may be, when this finding was applied to luteal tissues, it was found that the activity of diestrous corpora was increased approximately 45 per cent. By

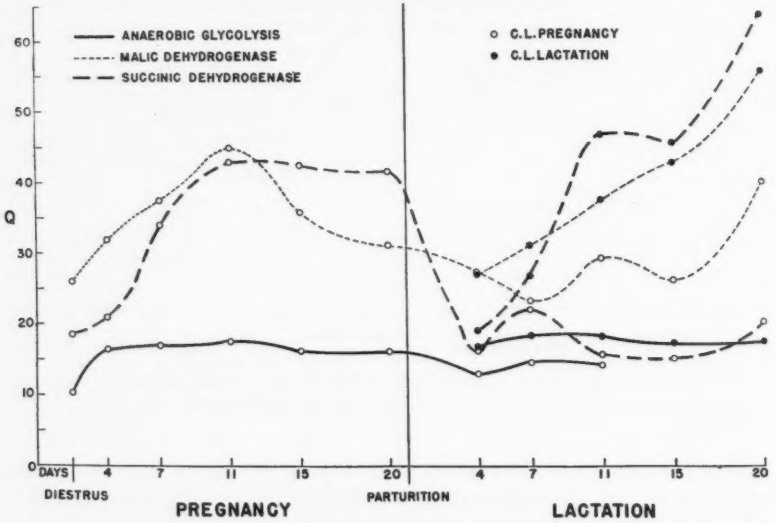

FIG. 2. The activities of the three enzyme systems, anaerobic glycolysis ($Q^{N_2}_{CO_2}$), malic dehydrogenase (Q_{O_2}) and succinic dehydrogenase (Q_{O_2}), of the corpora lutea of pregnancy and of the corpora lutea of post-partum ovulation.

contrast, the glycolytic activity of luteal tissues from various stages of pregnancy and lactation was activated 5 to 25 per cent by the addition of glutathione. With the addition of this tripeptide the variations of the glycolytic activity of the lutein tissue depicted in Figure 1 tend to be abolished and there is no significant change in the activity during the stages studied. If the *in vivo* glycolytic rate of luteal tissue is more accurately represented by the measured *in vitro* GSH-activated rate, then there is no essential change in the glycolytic activity during the formation, growth and regression of the corpus luteum.

It is of interest to compare the changes in activity of this anaerobic system in luteal tissue with the changes of the oxidative enzymes of carbohydrate metabolism which have been studied previously in this

laboratory (Meyer et al., 1947 and McShan et al., 1948). With the corpora of pregnancy the succinic dehydrogenase (SDH-ase) activity increases very little from diestrus to day 4 of pregnancy. From day 4 to day 11 the SDH-ase activity rises markedly to its peak and remains at that level until day 20 of pregnancy after which there is a rapid and maintained decrease. The SDH-ase activity of the corpora of lactation shows an over-all continuous increase from the 4th day of lactation to day 20. The malic dehydrogenase (MDH-ase) activity of the corpora of pregnancy shows a regular increase from diestrus to day 11. This activity is not maintained but falls off, reaching a low point at day 7 of lactation and rising to a high value again on the 20th day of lactation. The MDH-ase activity in the corpora of lactation increases regularly from day 4 to day 20. In contrast to these pronounced changes in activity of the enzymes of the aerobic oxidative cycle including cytochrome oxidase (McShan, Erway and Meyer, 1948), the anaerobic glycolytic activity of luteal tissue appears to be quite constant throughout pregnancy and lactation (see Figure 2).

There is some similarity between the changes in glycolysis and adenosinetriphosphatase (Biddulph, Meyer and McShan, 1946a) for the ATP-ase of the corpora of pregnancy does not change until shortly before parturition after which there is a marked increase in concentration. There is also no marked change in the ATP-ase of the corpora of lactation until day 20.

Consideration of the marked changes in the dehydrogenase activities in contrast to the stability of the glycolytic activity raises the question of substrate turnover of these complementary systems since one of the main pathways of pyruvate metabolism is through the Krebs tricarboxylic acid cycle. If the Q values for these three enzyme systems are recalculated in terms of micromoles of lactate (or pyruvate) produced and succinate and malate used, striking differences in the comparative activities of these enzyme systems are seen. These data are summarized in Table 2. It is worth noting that, in these systems, the two dehydrogenases have the ability to act on two to seven times as much substrate as that produced by the glycolytic enzymes.

That the amount of substrate produced by glycolysis *in vivo* might be even less than that calculated in Table 2 is indicated by studies of the Pasteur effect on lutein tissue. Determination of this effect showed approximately 30 per cent inhibition of glycolysis by oxygen (i.e., for corpora of day 20 of pregnancy: $Q_{CO_2}^{N_2} = 16.1$, $Q_{CO_2}^{O_2} = 11.7$). This finding would indicate that glycolysis may be less *in vivo* than that measured under anaerobic conditions and that the glycolytic scheme is actually contributing relatively less substrate to the Krebs cycle than that calculated in Table 2.

Since the substrate for the Krebs cycle in the scheme of **carbohydrate** metabolism is pyruvate derived from the activity of the gly-

colytic enzymes, it is tempting, on the basis of the above discussion, to postulate that the relatively high activity of the dehydrogenases in the luteal tissue indicates an extra-carbohydrate source of substrate for the Krebs cycle. Such a source is well known for tissues such as liver and kidney, but has not been demonstrated for ovarian tissues.

If the same relationship between the activity of the glycolytic and oxidative enzymes which is found *in vitro* holds true *in vivo* then it follows that compounds not produced by glycolysis in the lutein tissue are made available for the tricarboxylic acid cycle. It is possible

TABLE 2. THE RATES OF LACTATE PRODUCTION, SUCCINATE AND MALATE UTILIZATION BY LUTEAL TISSUE*

Stage	Micromoles of lactate produced		Micromoles of succinate used		Micromoles of malate used	
	Corpora of:		Corpora of:		Corpora of:	
	Preg.	Lact.	Preg.	Lact.	Preg.	Lact.
Diestrus	0.46		1.65		2.32	
Pregnancy days						
4	0.73		1.86		2.85	
7	0.75		3.02		3.34	
11	0.78		3.82		4.00	
15	0.71		3.78		3.19	
20	0.71		3.70		2.77	
Lactation days						
4	0.57	0.74	1.43	1.68	2.43	2.40
7	0.64	0.81	1.95	2.38	2.06	2.77
11	0.63	0.81	1.38	4.16	2.62	3.34
15		0.77	1.34	4.04	2.32	3.80
20		0.78	1.80	5.65	3.58	4.94

* Recalculated from the Q values in terms of micromoles per mg. dry tissue per hour.

that lactate of the blood is used by this tissue. However, it is more likely that the lutein tissue extracts such substances as amino acids and fatty acids from the blood as additional substrates for the Krebs cycle. It has been reported (Talbert, Stafford, Meyer and McShan, 1948) that there is a definite increase in the vascular pattern of the corpora during the latter part of pregnancy which correlates roughly with the increase in SDH-ase, MDH-ase and cytochrome oxidase activities.

In reference to the transport of extra-cellular substances into the lutein cells it should be pointed out that alkaline phosphatase of this tissue increases in late pregnancy (Stafford et al., 1947) and that in lutein tissue this enzyme is confined largely to the vicinity of the endothelium (Talbert et al., 1948).

The possibility also exists that the additional substrates for the tricarboxylic acid cycle may be derived in part from intra-cellular sources. Laqueur and Koets (1945) have shown that the total lipid content of the corpora lutea of pregnancy decreases from diestrus to day 16 of pregnancy and that there is a sharp rise in these lipids about the time of parturition. The protein content of the corpora follows the same general pattern. With reference to these changes it is noteworthy that the activities of both SDH-ase and MDH-ase are high in the latter part of pregnancy and drop off markedly at the time of parturition.

These additional data, when correlated with the previous discussion, provide a further basis for postulating that lutein tissue has the ability to metabolize fats and proteins.

There is an abundance of data available for comparing the changes in the activities of various enzymes during carcinogenesis. These studies have shown that regardless of the variable enzyme patterns of the parent tissues, the tumors arising from these tissues are characterized by a similar enzyme pattern. The present authors, however, are not in a position to make a comparison of the genesis and growth of luteal tissue to the genesis and growth of tumor tissue, since there are no data available concerning the enzyme systems in the follicular cell from which the lutein cell develops.

On the other hand, a justifiable comparison of enzyme pattern can be made between the rapidly growing luteal tissue and malignant tumors. As noted in the previous discussion, the potential glycolytic activity of luteal tissue during the phase of rapid growth is low while the activities of succinic dehydrogenase and malic dehydrogenase are relatively high during the same period. By contrast, a malignant tumor such as the Flexner-Jobling carcinoma possesses an entirely different type of enzymatic pattern. It has been shown (Novikoff et al., 1948 and LePage, 1948) that this tissue has a high glycolytic activity while enzymatic reactions characteristic of the Krebs tricarboxylic acid cycle, such as succinic dehydrogenase (Schneider and Potter, 1943) and oxalacetate oxidation (Potter, Pardee and Lyle, 1948), are low or negligible. It has been further shown that tumor homogenates do not have the ability to oxidize significant amounts of oxalacetate, even in the presence of a maintained high energy phosphate reservoir (Potter and LePage, 1949). Thus, it is evident that luteal tissue and tumors differ markedly in the enzymatic pattern concerned with oxidative metabolism even though they are both rapidly growing tissues.

SUMMARY

The anaerobic glycolysis of the lutein tissue and of the remaining ovarian tissue was determined during diestrus, pregnancy and lactation in the rat.

The glycolytic activity of the corpora of pregnancy increases sharply from the second day of diestrus to the 4th day of pregnancy and remains essentially unchanged throughout pregnancy. There is a significant drop in the activity of this tissue from day 20 of pregnancy to the 4th day of lactation. In the corpora of lactation, there is no significant change in activity.

The changes in the glycolytic activity of the lutein tissue are discussed in relation to the previously determined changes in succinic and malic dehydrogenases of this tissue.

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ON THE INFLUENCE OF 3-KETO-STEROIDS ON THE PHOSPHATASE CONTENT OF ATYPICALLY PROLIFERATING UTERINE EPITHELIUM IN THE GUINEA PIG

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INTRODUCTION

THE REMARKABLE findings of Huggins and his students (1941, 1943) with the increase of acid phosphatase in the blood serum in cases of prostatic cancer, and its decrease after castration or administration of estrogen, are amply known. The work of Huggins was preceded by that of Gutman and Gutman (1938) who were the first to report that development of prostatic epithelia dependent on, or induced by, testosterone was accompanied by an increase of acid phosphatase. These statements have been recently substantiated by new work of Huggins on the prostate under different experimental conditions (Pazos and Huggins, 1945; Huggins and Russel, 1946).

The question arose whether this specific action of androgen on the cellular enzyme pattern was exclusive of this group of steroids, or whether it was shared by all those steroids which are able to induce cellular proliferation. There was likewise the question whether the decrease of prostatic acid phosphatase as induced by estrogens was exclusive of this steroid, or whether this action was shared by other steroids able to counteract steroid-induced growth. If the above concept was true the phosphatase content should increase in estrogen-induced atypical uterine growth, and this increase should be antagonized by antiestrogenic 3-ketosteroids whose antitumorigenic actions have been under study for a long time (see summary Lipschutz et al., 1948).

As early as 1942 a project was elaborated, in association with Prof. Lipschutz, to study the phosphatase content in estrogen-induced epithelial and conjunctive growth and likewise the influence antiestrogenic 3-ketosteroids may have on the phosphatase content in similar growth. In preliminary work of Fuenzalida and Sarras[2] the

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TABLE 1*

Estrogen used	Absorbed per day μg.	Duration of exper. days	Weight of uterus grams	Number of animals	Phosphatase units (average)				No. of animals with alkal. phosphat. above upper limit	
					Mucosa		Myometrium†			
					Alkal.	Acid	Alkal.	Acid	Mucosa	Myometrium
0	0	0		28	54‡	27§	31	6.3	0	0
benzestrol diprop.	2 to 4 (injections)	37–101 (73)	1.8–19.0 (5.8)	*37*	119	18	56	5	*23*	5
diethylstilbestrol	280 (subcut. tabl.)	63– 90 (73)	2.1– 9.8 (4.3)	*31*	80		62	—	*12*	5

* Comprises experiments made in collaboration with Drs. M. Pinto, R. Nahmías, A. Santa-María, C. Díaz and J. Tenorio, working for their M.D., Universidad de Chile, 1946 to 1948.

† Or entire uteri; 28 animals with the determination of alkaline phosphatase; 23 animals with the determination of acid phosphatase.

‡ 12 animals.

§ 7 animals.

following results were obtained: (1) there was a considerable increase of the alkaline phosphatase content of the uterus in guinea pigs to which different estrogens were administered for several months and in which an increase of the uterine weight had taken place; (2) there was no increase of acid phosphatase in the uterus; (3) there was no increase of alkaline or acid phosphatase in estrogen-induced uterine or extrauterine abdominal fibroids; (4) when experiments were prolonged for 3 months or more and excessive uterine weights of 12 g—instead of 1 g in the normal animal—were obtained, the alkaline phosphatase content began again to decrease. On the basis of the statements (1) and (3) the following two tentative suggestions were made to explain the decrease in experiments of long duration: (a) that there was no notable increase of alkaline phosphatase in the growing myometrium as there was none in fibroids, and that the increase of uterine alkaline phosphatase was due predominantly to the proliferating uterine epithelium, in such a way that with the considerable growth of the myometrium the alkaline phosphatase content of the epithelium *seemingly* again decreased; (b) that the alkaline phosphatase content of the uterine epithelium *really* decreased under the influence of the epitheliotoxic action of estrogen so often observed in similar experiments. To prove these suggestions, phosphatases were determined in all subsequent work separately in the myometrium and in the epithelium removed, together with the submucosa, from the myometrium.

METHODS

The uterus was weighed and opened by scissor or knife. The epithelium together with the submucosa (to simplify: "mucosa") was separated by scraping with a blunt scalpel. The myometrium and mucosa were weighed separately. The myometrium was triturated; distilled water was added so as to obtain a concentration of 1 mgm of myometrium, or of mucosa, per 1 ml. The material was kept at 4°C for 24 hours. The method of King and Armstrong (King et al., 1942) was used for the determination of phosphatase (disodium-phenyl-phosphate as substrate). Readings were made with Klett's photocolorimeter with filter 66. All data refer in the following to King-Armstrong units per gram of fresh tissue. In our former work with Sarras, the method of Kay (Kay, 1930) was used and for this reason these results are omitted in the present paper.

Influence of estrogen on the phosphatase content of myometrium and mucosa

Results with 91 castrated guinea pigs are given in table 1.

In our attempts to separate the myometrium and mucosa of normal uteri we often failed; there were only 12 animals with the separated etermination. Results with the entire uterus and the myometrium were pooled together. The highest values obtained with the separate determination in the mucosa and myometrium was taken as

TABLE 2

Stilbestrol absorbed per day μg.	3-ketosteroids		Duration of exper. days	Weight of uterus gr.	No. of animals	Mucosa		Myometrium		No. of animals with alkal. phosphat. above normal upper limit	
	used	absorb. per day				Alkal. phosph. un.	Acid phosph. un.	Alkal. phosph. un.	Acid phosph. un.	Mucosa	Myo-metrium
280	0	0	63–90 (73)	2.1–9.8 (4.3)	31	80	—	62	—	12	5
214	Progester.*	213	30–150 (92)	1.0–2.8 (1.7)	23	39	26	39	9	2	2
245	Progester.†	45	54–93 (77)	1.0–4.6 (2.44)	30	32	—	49	—	0	3
296	Testost. propion.	265	61–63 (62)	2.8–9.9 (4.3)	6	26	—	37	—	0	0
237	Dihidrotestost.	82	35–153 (86)	0.6–5.2 (3.0)	21	49	46	63	13	2	3
190	Desoxycorticost.	198	30–124 (83)	1.5–8.8 (3.0)	21	107	21	36	6	9	1

* Tablets of pure progesterone.
† Tablets containing but 40% of progesterone.

the normal upper limit which was for alkaline phosphatase of the mucosa 87 and of the myometrium 89.

Benzestrol dipropionate and diethylstilbestrol were used as estrogens. The first was administered by subcutaneous injection of oily solutions; injections of 5 and 10 μg of benzestrol were given thrice weekly. Stilbestrol was absorbed from subcutaneously implanted tablets containing 90% of stilbestrol and 10% of cholesterol. The animals were killed after a treatment of 1 to 3 months. With benzestrol the alkaline phosphatase content of the mucosa surpassed the upper limit of normal uteri in no less than 23 out of 37 cases; with stilbestrol in 12 out of 31 cases. There was no increase of acid phosphatase in the mucosa. There were only 5 out of 37 animals and 5 out of 31 animals with an increase of alkaline phosphatase in the myometrium. Determination of acid phosphatase in the mucosa and of both phosphatases in the myometrium were regularly made in subsequent work without an increase of acid phosphatase and rarely an increase of alkaline phosphatase being noted; consequently the respective data will be omitted here.

We have referred in the Introduction to the decrease of uterine alkaline phosphatase in experiments with the prolonged action of estrogen. Results with benzestrol (fig. 1) show clearly that there was a gradual decrease even when the mucosa was examined separately; the number of animals beneath the normal upper limit was much greater at 3 months than at 40 to 50 days. Less evident were the results with stilbestrol (fig. 2) probably due to not disposing of experiments of 40 to 50 days for comparison. The results speak in favor of the concept that with the more prolonged action of estrogen the alkaline phosphatase shows a tendency to diminish again. Indeed, the objection can be made that in experiments of longer duration there was a greater development of loose or oedematous conjunctive tissue and this may have stultified the results.

Influence of antiestrogenic steroids on the estrogen-induced increase of the alkaline phosphatase content

Results with 101 animals are summarized in table 2.

In all experiments stilbestrol absorbed from subcutaneously implanted tablets was used. The antiestrogenic steroids also were absorbed from similar tablets. In some cases tablets containing only 40% of the specific steroid were used.

With *progesterone* the antiestrogenic actions as described in former work of Lipschutz and his students became evident: closure of the vagina, decrease of uterine weight, prevention of fibroids, but no inhibition of mammary growth. Only in 2 out of 53 animals the normal upper limit has been surpassed (fig. 3). Similar results were obtained with *testosterone propionate* and *dihydrotestosterone:* the normal upper limit has been surpassed in only 2 out of 27 animals.

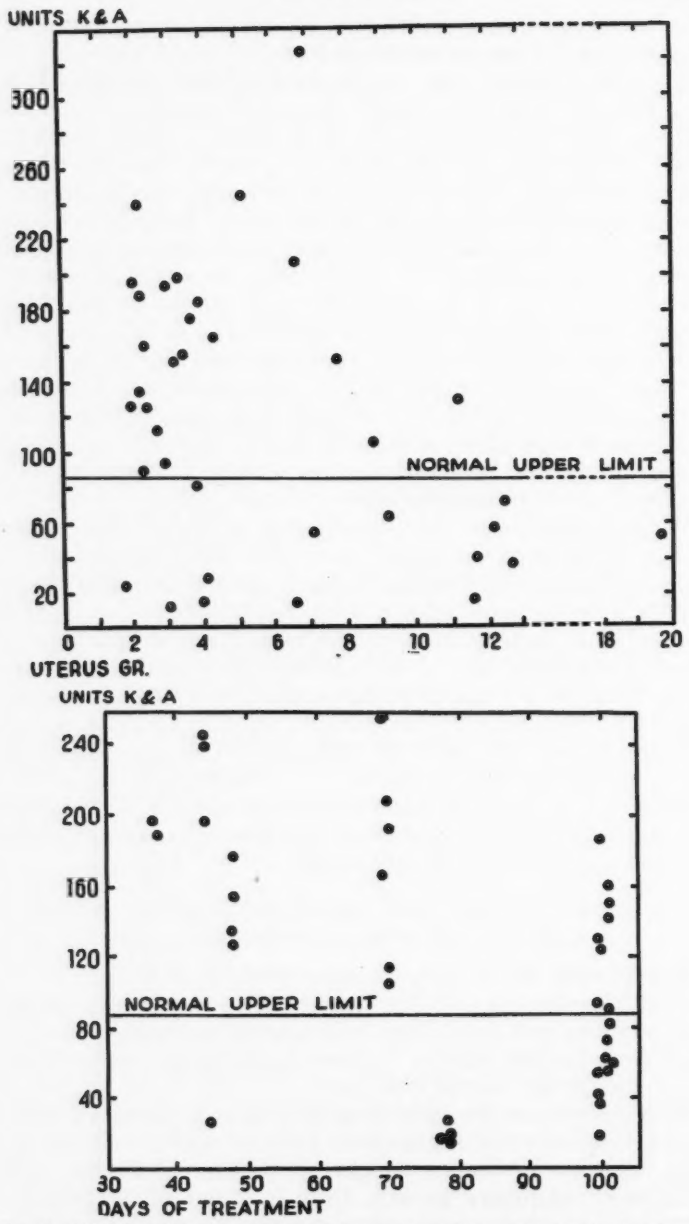

FIG. 1. Alkaline phosphatase in uterine mucosa (or submucosa) of guinea pigs treated with benzestrol dipropionate. King & Armstrong units per gram.—A. The greatest frequency of increase is in uteri of 2 to 5 g.—B. The greatest frequency of increase is at 37 to 48 days. At 78 days, and specially at 100 days, there is again a considerable number of animals with normal values.

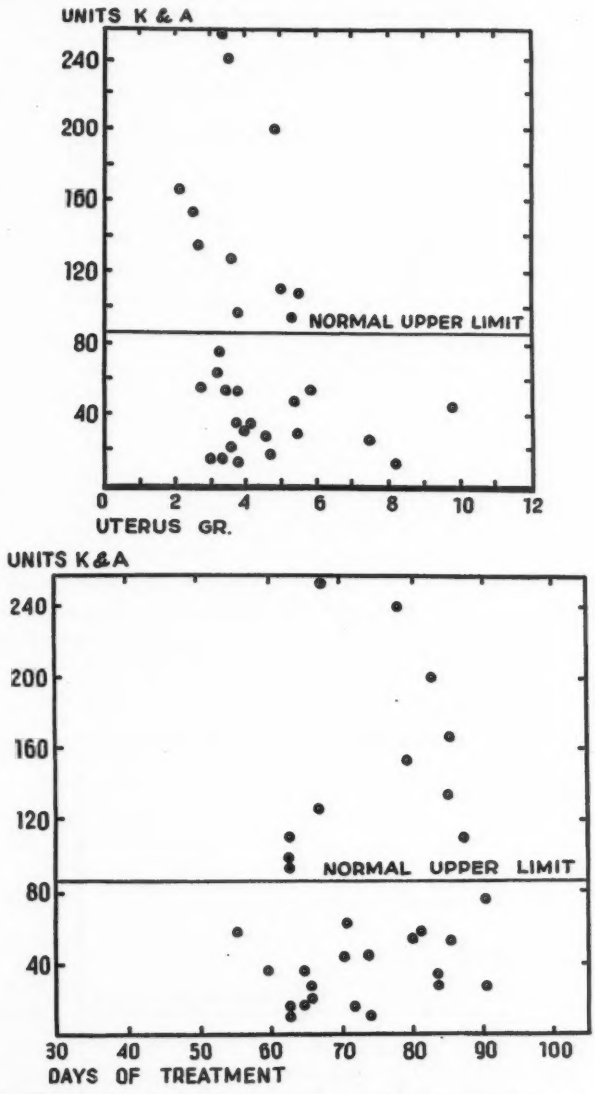

FIG. 2. Alkaline phosphatase in uterine mucosa (or submucosa) in guinea pigs treated with diethylstilbestrol. For details see text.

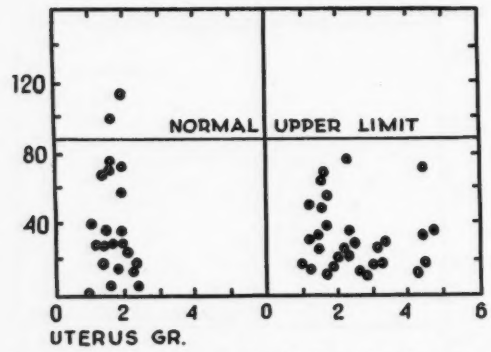

FIG. 3. Influence of progesterone on the alkaline phosphatase content of uterine mucosa (or submucosa) in animals receiving simultaneously stilbestrol. *Compare to fig. 2A.*

The difference between the stilbestrol group, on the one hand, and the groups with stilbestrol and antiestrogenic steroids on the other hand was striking. The percentage of animals above the normal upper limit with stilbestrol alone was about 40, with progesterone or androgens about 4 to 8. Inhibition of estrogen-induced increase of alkaline phosphatase with androgens was less pronounced than with progesterone (fig. 4). This was concomitant with a less pronounced antifibromatogenic action; there were various animals with fibroids.

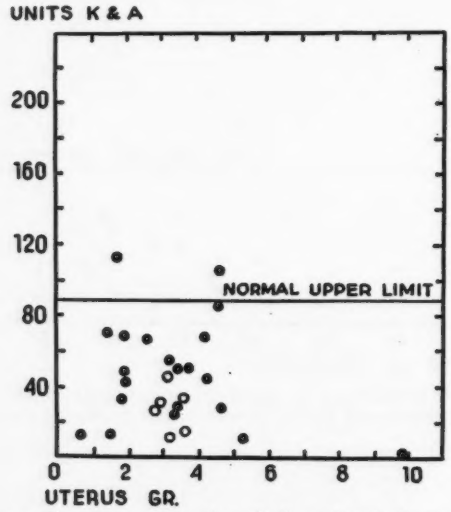

FIG. 4. Influence of androgens on the alkaline phosphatase content of uterine mucosa (or submucosa) in animals receiving simultaneously stilbestrol. ○ testosterone propionate; ● dihydrotestosterone. *Compare to fig. 2A.*

Contrary to our expectation no decrease of alkaline phosphatase was obtained with desoxycorticosterone. This was probably due to the quantities absorbed having been insufficient; fibroids were prevented but the vagina remained open.

DISCUSSION

When discussing the quantitative results with the action of different steroids as given in tables 1 and 2 it seems preferable to consider the relative number of animals in which the phosphatase content increased above the normal level, instead of comparing averages. There was in our work, as exemplified by figures 2 and 4, no frequency

TABLE 3

Substances used	No. of animals total (n)	Above normal upper limit (z)	Percentage ($p=\frac{z+1}{n+2}\cdot 100$)	σ of the percentages*
Stilbestrol	31	12	39	8.3
Stilbestrol and progesterone	53	2	5	2.9
Stilbestrol and testosterone propionate	6	0	13	11.2
Stilbestrol and dihydrotestosterone	21	2	13	6.8
Stilbestrol and desoxycorticosterone	21	9	43	10.1

* $\sigma p=\sqrt{\frac{p(100-p)}{n+3}}$.

distribution around a statistical mode but very disperse values. Under these circumstances we must center the discussion around the question whether the differences between the percentages of animals above the normal upper limit are significant.

Percentages should be calculated according to the formula $p=\frac{z+1}{n+2}\cdot 100$, in which p is the corrected percentage, z the number of positive cases and n the total number of cases (Van Der Waerden, 1936). Results are given in table 3.

The difference between the stilbestrol group and the stilbestrol-progesterone group is striking, and so also the difference between the stilbestrol and the stilbestrol-androgen groups. There can be no doubt that progesterone and so also androgens counteract the estrogen-induced increase of alkaline phosphatase in the uterine mucosa. An even more striking picture of this action of the mentioned steroids is obtained when only those experiments are compared in which uterine weights were equal. Such a selective comparison is made in table 4.

Our results may be compared to those which have been obtained in recent years with cytochemical studies of the phosphatase content in the uterus. Atkinson and Elftman (1946, 1947) stated an increase of alkaline phosphatase in the uterus of castrated mice with the administration of estradiol benzoate during three days; there was an increase not only in the endometrium but also in the myometrium and especially in the longitudinal layer. Dempsey et al. (1949) found that alkaline phosphatase disappeared completely in the uterus of the rat 26 days after hypophysectomy and that the phosphatase content was restored to normal when whole pituitary powder was given.

TABLE 4

Group	No. of animals		Percentage above normal upper limit	Significative difference*
	Total	Above normal upper limit		
Stilbestrol	19	7	38	
Stilbestrol and progesterone	53	2	5	3
Stilbestrol	31	11	37	
Stilbestrol and testosterone propionate	6	0	13	2
Stilbestrol	23	8	36	
Stilbestrol and dihydrotestosterone	21	2	13	2

* $D = \frac{p_1 - p_2}{\sqrt{\sigma_{p_1} + \sigma_{p_2}}}$. Values of D equal to or above 2 are considered significant.

Atkinson and Engle (1947) reported an increase of alkaline phosphatase in the endometrium of women and M. rhesus; it was remarkable that the increase was limited to the proliferative or estrogenic phase, there being a decrease in the secretory or luteal phase. On the other hand, there was always an increase in the work of Atkinson and Elftman (1946) with estrogen in mice when progesterone or testosterone propionate were added. This is contrary to our results with progesterone. But this might have been due to quantities too small or to a duration too short. It must also be held in mind that in our work we are dealing with atypical proliferation of the endometrium; indeed it does not seem very likely that there should be such a definite difference as to the influence of progesterone in typical and atypical proliferation of the same cells.

All these findings are acquiring gynecological interest as evidenced by the work of Zondek and Hestrin (1947) on the phosphorylase content in the endometrium of primates and women and its bearing to sterility. Arzac and Blanchet (1948) have studied the alkaline phosphatase content and glycogen in endometrial biopsies.

SUMMARY

A considerable increase of chemically determined alkaline phosphatase took place in the uterus of the guinea pig under the influence of a prolonged treatment with estrogen.

This increase was localized mainly in the greatly hypertrophied uterine mucosa, or submucosa.

The alkaline phosphatase content of the uterine mucosa, or submucosa, may become several times that of a non treated animal.

When experiments were prolonged for several months, alkaline phosphatase showed the tendency to decrease again.

When progesterone was administered simultaneously with estrogen the alkaline phosphatase increase in the mucosa, or submucosa, was counteracted so as to attain, under certain quantitative conditions, the same level as in non treated animals.

A similar though less pronounced anti-phosphatase action was observed with androgens as testosterone propionate and dihydrotestosterone. No such action was found with desoxycorticosterone.

There was only slight increase of alkaline phosphatase in the myometrium when greatly hypertrophied under the influence of the prolonged treatment with estrogen.

No increase of acid phosphatase took place in any part of the uterus under the influence of estrogen.

There was no increase of alkaline or acid phosphatase in estrogen-induced uterine or extrauterine abdominal fibroids.

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THE FETAL BOVINE THYROID: MORPHOGENESIS AS RELATED TO IODINE ACCUMULATION[1]

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In the fetal calf, which has a gestation period of approximately 285 days, thyroxine-like iodine can be detected in the thyroid gland on the sixtieth day of intrauterine life. Thereafter, the amounts of thyroxine-like and total iodine in the gland increase steadily, and the rates of increase of these two iodine fractions have been shown to be related exponentially to fetal body weight, length, and age (Wolff *et al.*, 1949). Since, in addition, the *concentration* of iodine in the developing gland (i.e., mg. of iodine per 100 gm. fresh tissue) appeared to increase progressively, it was postulated that with increasing age, the fetal thyroid acquired an increased capacity for iodine storage.

In the present investigation, we have attempted to correlate the morphogenesis of the gland with its iodine content. Since the follicle might be regarded as the structural and functional unit of the gland, we sought to determine the relation of its development to the capacity of the gland to store iodine.

EXPERIMENTAL

Fifty-nine thyroid glands were collected from fetal calves ranging in age from 53 to 265 days in intrauterine life (gestation 278–285 days). The age of each fetus was determined as described in an earlier report (Nichols *et al.*, 1949). The methods employed for iodine determinations have also been described (Wolff *et al.*, 1949). The glands were fixed in Bouin's fluid, embedded in nitrocellulose, and sectioned at six micra. The sections were stained with hematoxylin and eosin and also by the Malloray-Azan method.

RESULTS

The thyroid glands have been grouped according to stages of histogenesis as shown in Table 1. At the earliest stage (53 to 70 days), the glands consisted of proliferating masses of branching epithelial

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plates or cords, one to three cells wide, surrounded by well developed blood and lymphatic capillaries (Plate I, fig. 1). The interstitial mesenchymal connective tissue was not abundant at that time, except in the center of the lateral lobes or in the isthmus. Many nuclei were undergoing mitotis, indicating rapid growth of the epithelial cords. The individual cells adhered to one another, but were sharply outlined. The abundant, lightly staining cytoplasm of these cells contained no visible colloid droplets.

Colloid droplets (staining with either aniline blue or with orange G) first made their appearance in the cell cytoplasm of thyroid glands in 75- to 88-day-old fetuses. These droplets moved toward the cell

TABLE 1. IODINE CONTENTS OF FETAL THYROIDS

Estimated age range of group	Number of glands examined		Average iodine values mg. per cent			Reference to figures (Plates I–III)
	Histologically	Chemically	Thyroxine	Non-thyroxine	Total*	
days						
53–70	7	4	3.0	14.1	17.1 ± 5.5	1
75–88	6	12	4.0	7.3	11.3 ± 1.5	2–6 and 9
90–105	14	14	2.9	9.6	12.7 ± 2.0	10
106–118	6	9	6.3	11.6	17.9 ± 3.1	11
120–133	4	6	5.5	14.5	20.0 ± 4.5	12
135–152	7	11	8.5	24.0	32.6 ± 2.9	13
153–172	5	13	10.8	23.3	34.1 ± 4.8	14
175–205	3	4	19.1	48.0	67.0 ± 7.1	15
218–265	5	8	35.1	82.4	118.0 ± 15.0	16

* Standard error of mean $= \sigma_{\bar{x}} = \sqrt{\frac{\sum_{i}^{n}(x_i - \bar{x})^2}{n(n-1)}}$.

border and eventually passed into intercellular spaces marking the center of the future follicle (figs. 3 and 4).

Steps in the development of true follicles are clearly shown in figs. 4, 5, and 6. During the follicle formation, the regularity of cord arrangement became less distinct (fig. 2).

Photomicrographs 9 to 16 (all of the same magnification) show the gradual development of the thyroid parenchyma. In the process of reconstruction into follicles, the mass of epithelial cords (now constituting interfollicular tissue) gradually diminishes and, in the later stages of thyroid development, this interfollicular tissue is represented by rather small islets of cells. At the periphery of the gland, or, in later stages, at the periphery of the lobules, the cord arrangement remains as an uninterrupted rim up to 150th day of intrauterine life. This tissue apparently serves as a reserve material for the growing thyroid since numerous mitoses and very young follicles are present.

Intracellular colloid formation and growth of the follicles were not appreciable for a period of approximately 60 days after their first appearance. There also was no major increase in total iodine content in the glands at that time. As gestation progressed, however, the rate of follicular development clearly increased, as did the amounts of colloidal material in the lumina of the follicles, and the intracellular colloid droplets in the follicular epithelium. This intracellular activity accelerated notably during the last 2–2½ months of pregnancy. At that time, fetal thyroids reached almost complete development and a distinct increase in total iodine content was recorded. The epithelial cells which now had attained their maximum size were mostly columnar. Nuclei were round or oval in shape and vesicular in character. The apical portion of the cytoplasm of the follicular cells of many follicles was filled with numerous discrete colloid droplets (figs. 7 and 8). These figures also show the manner in which colloid droplets pass into the follicular cavity. This picture of secretory activity was not unlike that described by De Robertis in his studies on colloid secretion by the follicular epithelium (1941, 1942, and 1949).

DISCUSSION

In fetuses ranging from 53 to 70 days in age, the thyroid contained about three mg. per cent thyroxine and about 17 mg. per cent total iodine. In contrast, the concentrations of these two iodine fractions in the livers of three fetuses aged 62, 68, and 70 days—though too low to permit an accurate quantitative measure—were less than one-tenth those of their respective thyroid glands (Wolff and Nichols, 1949).

Explanation of Plates

Photomicrographs were taken with 4 mm. objective (figs. 1 and 2), with oil immersion objective (figs. 3 to 8), and with 16 mm. objectives (figs. 9 to 16). Magnification ×400, ×750, and ×150 respectively.

Fig. 1. An area from the central region of the thyroid gland of 63-day-old calf fetus. Note the arrangement of cells into quite regular cords. No intracellular colloid or follicles are present.

Fig. 2. An area from the thyroid gland of 78-day-old calf fetus. Note a few very young follicles. Also note that the cord arrangement became less regular.

The following figures (3 to 6) show initial stages of development of the follicles.

Fig. 3. Individual colloid droplets stainable with aniline blue or orange G can be seen in the cytoplasm (arrow).

Fig. 4. First drop of colloid located between two oblong, neighboring cells of the cord (arrow).

Fig. 5. Young follicle showing individual colloid drops not yet coalesced. Also note that one droplet of colloid is passing from the apical region of the cell into the lumen; note indented nucleus.

Fig. 6. Young follicle having many characteristics shown in fig. 5 but containing faintly vacuolated, irregular mass of fusing colloid droplets.

Fig. 7. Follicular epithelium from near-term gland showing two cells apical portions of which are ready to be cast off into the lumen.

Fig. 8. High columnar epithelium from the thyroid of fetus near-term showing massive accumulation of stainable colloid droplets in the apical zone of the cytoplasm and their passage into the lumen.

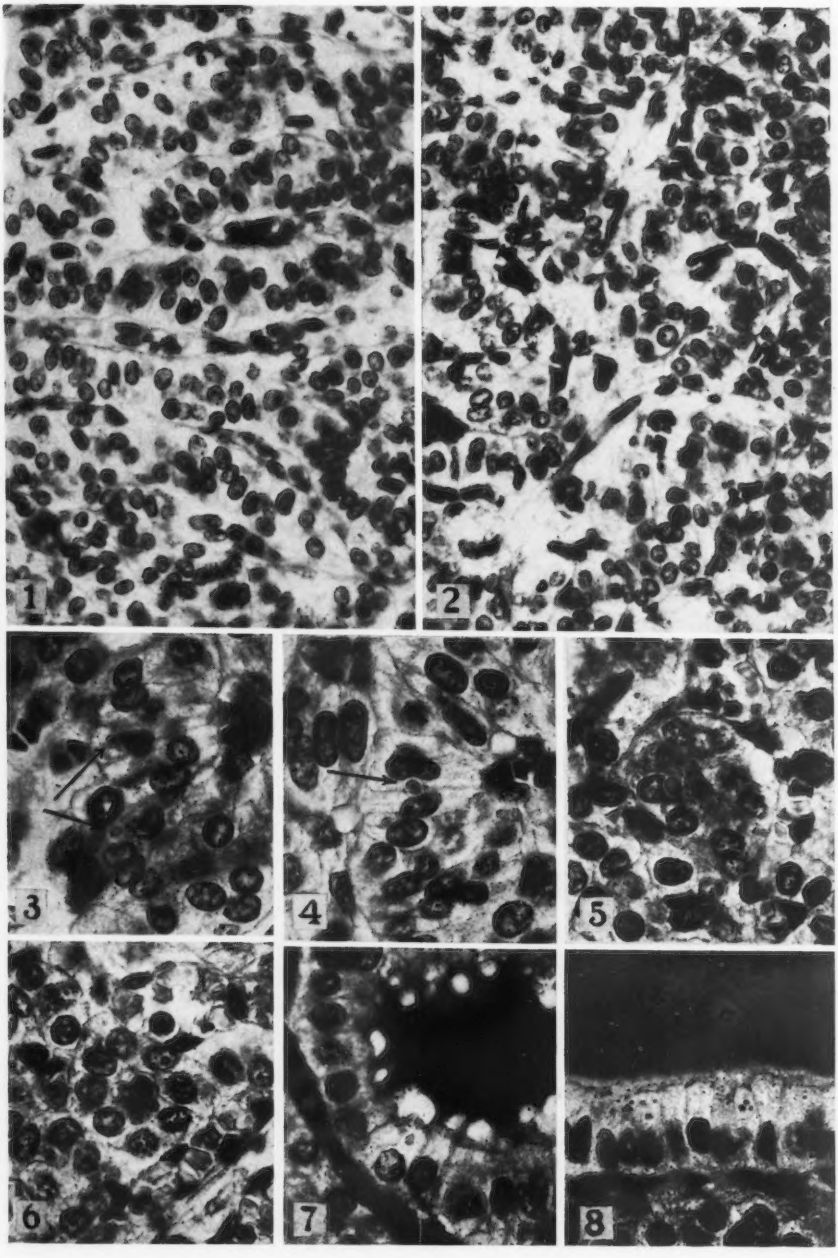

PLATE I

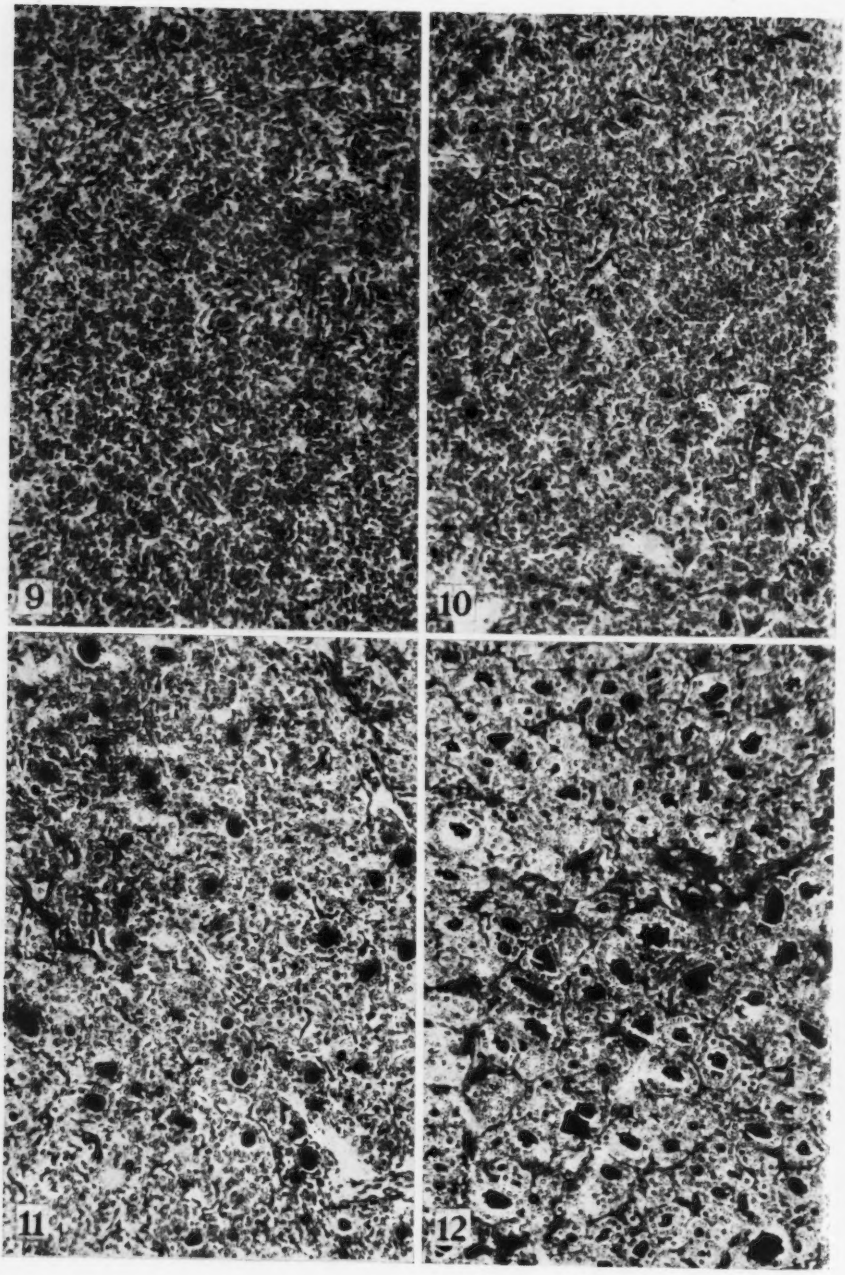

PLATE II

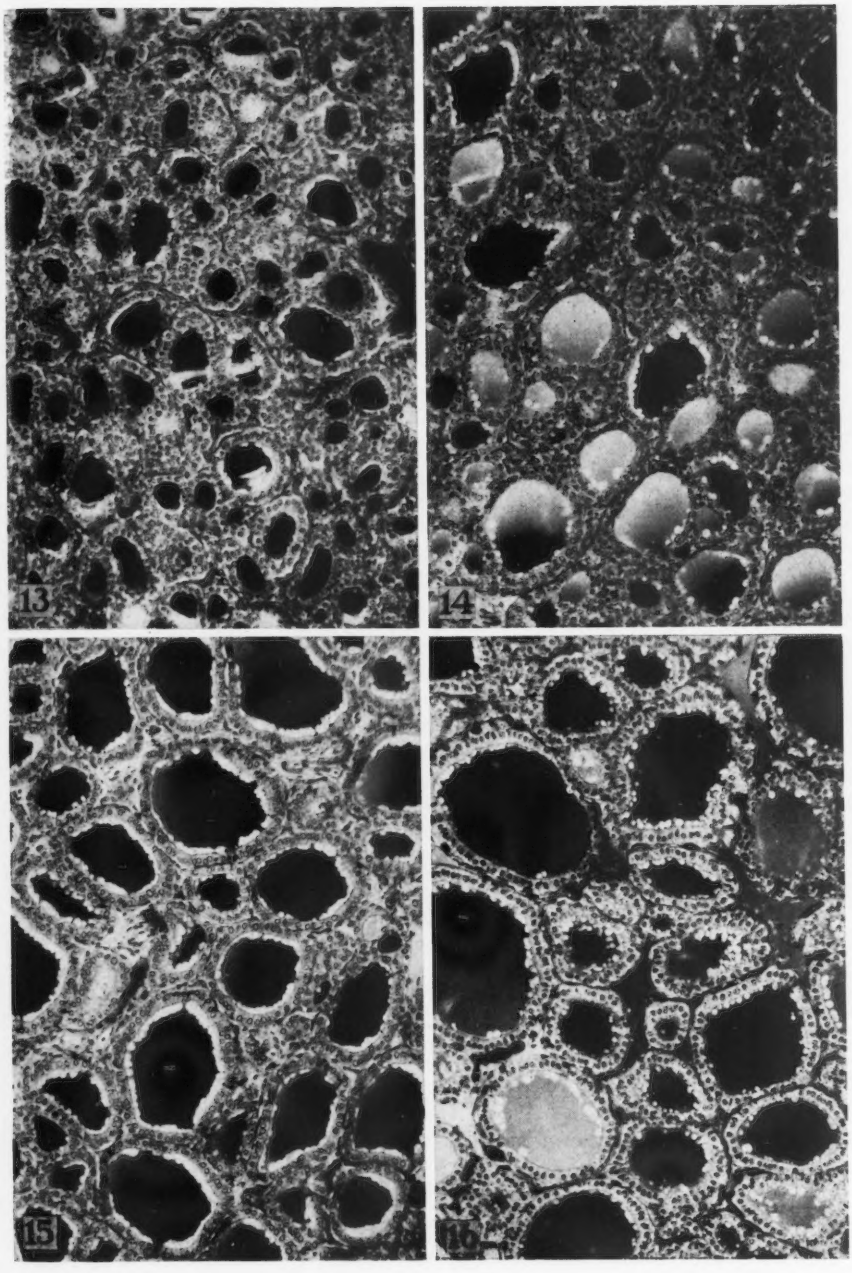

PLATE III

FIGS. 9 to 12 on Plate II and figs. 13 to 16 on Plate III are photomicrographs representing thyroids of calves, 88, 90, 108, 125, 135, 155, 175, and 254 days of intrauterine life respectively. Note progressive numerical increase of typical thyroid follicles and their development accompanied by growth and development of follicular epithelium. Also note gradual diminution of interfollicular nests of cells.

Thus concentration and storage of organic iodine in the fetal thyroid tissue takes place not only while the gland is still devoid of its characteristic architecture, but even prior to any histological manifestation of this specific function (intracellular colloid droplets). Follicular arrangement of thyroid epithelium is, therefore, not a prerequisite to iodine storage by this tissue.

Similar findings have been reported for the fetal pig by Rankin (1941) who detected thyroxine and diiodotyrosine in the thyroids of 52-day-old fetuses (gestation period 114 days) at a time when their thyroids contained neither follicles nor colloid.

Hogben and Crew (1923) reported follicle differentiation and minute colloid masses at three months in bovine fetal glands. In the same species, Aron (1931) found small amounts of stainable colloid at 15–18 cm. stage (corresponding to about 90 days of pregnancy as calculated here), whereas Abbott and Prendergast (1937) described colloid and follicle formation during the second month of life. In our series of calf thyroids, colloid was first detected *within the cytoplasm* of individual cells and *in the lumen* of very young follicles on the 75th day of intrauterine life.

The following may be considered as the first stages of the histological manifestation of secretory activity in early fetal thyroids: a) the appearance of intracellular colloid droplets and beginning of follicle formation in the glands of about 75- to 88-day-old fetuses; b) the presence of completely formed, although very small, follicles at approximately 90 days. In spite of this definite advance in their histological development, the fetal thyroids showed no major changes in the amounts of iodine stored, either during this period or in the following few weeks.

In the following months (from 120 to 205 days), the increase in the concentration of organic iodine was accompanied by the gradual histological development shown in figs. 12 to 15. Histological manifestations of activity, as judged by production and storage of colloid, in glands of these age groups differed only in degree and not in general character. The average values for total iodine were 20.0, 32.6, 34.1, and 67.0 mg. per cent for the groups represented by photographs 12 to 15, respectively. In the final months of gestation, total organic iodine content reached its maximum, averaging now 118 mg. per cent. This increase in storage capacity of the thyroid gland was accompanied by a very marked rise of functional activity of the follicular cells.

SUMMARY

Thyroxine-like and other organically bound iodine compounds appear in the fetal calf thyroid between the 53d and 70th days, a time prior to the appearance of histologically detectable intracellular colloid or follicle formation, which takes place at 75–88 days. Since the livers of fetuses aged 62–70 days contain practically no iodine,

the presence of these organic iodine compounds was considered a manifestation of iodine storage by the gland.

From the 75th to 118th day, the predominating features of the developing thyroid were the appearance of colloid and differentiation of the parenchyma without a pronounced increase in the iodine content of the gland.

Between the fourth and seventh month, gradual development of the fetal thyroid gland was accompanied by a steady increase in its organic iodine content.

An appreciable increase in organic iodine content occurred near term, and was accompanied by definite histological manifestations of increased functional activity of the follicular epithelium.

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THE NECESSITY OF TESTIS AND THYROID HORMONES FOR THE MAINTENANCE OF THE SEROUS TUBULES OF THE SUBMAXILLARY GLAND IN THE MALE RAT

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SINCE the time, about a century ago, when research on the salivary glands began in earnest, most of the investigations dealing with these organs indicated that they were under the control of the nervous system (Babkin, 1944). Therefore, it came as a surprise to learn that the male sex hormone plays an important role in the maintenance of the serous tubules of the submaxillary glands of mice. This was first suspected by Lacassagne (1940a) when he found that this structure was far better developed in male than in female mice. Later he showed that the extirpation of the testes produced the atrophy of the serous tubules, while testosterone injections restored them to a normal condition (Lacassagne, 1940b). These effects were accompanied by changes in the enzymatic content of saliva. Thus, the ability of saliva to liberate glucose from starch was greater in males than in females, decreased by castration in males, and increased by testosterone injections in females (Raynaud and Rebeyrotte, 1949a, 1949b).

A complication was introduced by the finding that hypophysectomy produced a more pronounced atrophy of the serous tubules than castration (Lacassagne and Chamorro, 1940). This was explained by Raynaud (1946, 1947) on the assumption that cortico-adrenal hormones also can stimulate the serous tubules to some extent. Thus, after hypophysectomy, the absence of both adrenal and testicular secretions would account for the more complete atrophy of the tubules than after castration. However, the data of this author indicated that the role of the adrenals was only slight if any. Other endocrines might, therefore, be involved.

A role of the thyroid gland seemed likely, since in the rat thyroidectomy produces an atrophy of the serous tubules of the submaxillary gland (Leblond and Grad, 1948a). To study the roles of testis and thyroid, these two glands were removed and substitution therapy with testosterone or thyroxine or both was instituted. In this manner, it was demonstrated that a joint action of these hormones is necessary to maintain a normal development of the serous tubules in the submaxillary gland of the rat.

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METHODS

The submaxillary gland of the adult male rat is rather complex: the acini, which may be classified as mucous,[1] are joined by short intercalated ducts to tubules lined by cells full of serous granules (Figs. 1 and 2). These serous tubules in turn empty into excretory ducts. Both tubules and ducts stand out in the sections because of their acidophilic properties, but the tubules may be differentiated from the ducts by their smaller lumen, the basal position of the nucleus in their cells and especially the presence of numerous granules in their cytoplasm.

Preliminary experiments were designed in order to examine how the serous tubules were affected by feeding and fasting, by age and sex and by thyroidectomy. The results of this work led to the main series of experiments, in which the effect of testosterone and thyroxine were examined in castrated-thyroidectomized rats.

Preliminary experiments

The influence of feeding and fasting was examined in six groups of adult male rats which were fasted from 11 P.M. one evening to 8 A.M. the next

Table 1. The effect of feeding and fasting on the serous tubules of the submaxillary gland of the male rat

Hours of fast	Average body weights (gm)		Average diameter of Serous tubules (micra)
	Initial	Final	
0	294	—	45.7 ± 2.0
2	297	—	51.0 ± 1.8
8	297	—	47.0 ± 2.3
30	295	273	48.0 ± 2.0
120	298	239	44.7 ± 0.2

Statistical Analysis: Differences between these values were not significant, except between the 2 and 120 hour groups ($P = 0.02$). The difference between the 0 and 2 hour groups ($P = 0.10$) was near the borderline of accepted significance.

morning, when they were fed Purina fox chow ad libitum for one hour. One group of 4 animals was sacrificed at the end of this feeding period. The other animals were then fasted again, and sacrificed in groups of 4 or 5 animals at 2, 8, 30 and 120 hours later. The submaxillary glands of the animals were fixed in Susa, sectioned and stained with hematoxylin-eosin. The diameters of the serous tubules were measured in the sections with the help of a micrometer placed inside the ocular of the microscope. The diameter was taken as the maximal width of each tubule, that is to say, the longest perpendicular to the longest axis of the cross sections of a tubule. Ten measurements were averaged per animal; grand averages were then calculated for each group and reported in Table 1.

[1] The acini of the submaxillary gland of the adult male rat, when fixed in biochromate-formol, gave an intense periodic acid-leucofuchsin reaction and stained metachromatically with toluidin blue, They were, therefore, considered as being mucous and will be thus referred to in the present article.

It must, however, be noted that Stormont (1932) and many others considered that these acini did not consist of true mucous cells, but rather of "special serous cells" or "tropochrome cells."

The effect of age and sex was examined by measuring the diameter of the serous tubules in the submaxillary glands of the following groups of rats: 1) Five males and two females, 10 day old; 2) four females and four males, 39 day old; 3) nine males and nine females, 150 day old. The animals were fed on Purina fox chow (with the exception of the suckling 10 day old ones). They were sacrificed with chloroform. The submaxillary of the animals of groups 1 and 2 were fixed in Orth and stained with hematoxylin-eosin. The animals of group 3 were the normal controls of the next experiment. The diameters of the serous tubules were measured as in the previous experiment and reported in Table 2.

The effect of thyroidectomy and thyroxine treatment was examined in the following groups of rats, which all weighed less than 45 grams at the time of operation: 1) nine normal control males; 2) sixteen thyroidectomized males; 3) nine normal control females; 4) nine thyroidectomized females. It may be

TABLE 2. THE EFFECT OF AGE AND SEX ON THE SEROUS TUBULES OF THE SUBMAXILLARY GLAND OF THE RAT

Age (Days)	Average diameter of the serous tubules (micra)	
	Males	Females
10	22.3 ±0.52	21.3 ±0.58
39	31.9 ±0.61	29.4 ±0.45
150	45.8 ±0.48	42.3 ±0.35

Statistical Analysis: All differences between any two groups (except between 10 day old males and females) are highly significant ($P < 0.001$).

noted that thyroid removal included the ablation of the parathyroid glands.

The animals were then fed on Purina fox chow and left untreated for the next 16 weeks. At that time, the thyroidectomized animals were subdivided into two sub-groups, one of which remained untreated, while the other received 7.5 micrograms of sodium-DL-thyroxine twice daily for the next two weeks. All animals were then sacrificed with chloroform. The submaxillary glands were fixed in Susa and stained with hematoxylin-eosin. The diameters

EXPLANATION OF PLATE I

FIG. 1. A section of submaxillary gland of normal adult male rat. The section was fixed in Susa, and stained with hematoxylin-eosin . ×500

This shows a duct (upper left hand side) leaving two tubules (lower centre and right), the remainder of the field being made up of mucous acini. This figure shows some of the differences between serous tubules and ducts: viz., the tubules have more basally located nuclei, narrower lumina and more granular cytoplasm.

FIG. 2. A section of submaxillary gland of normal adult male rat. The section was fixed in Susa, and stained with H & E. ×1,100

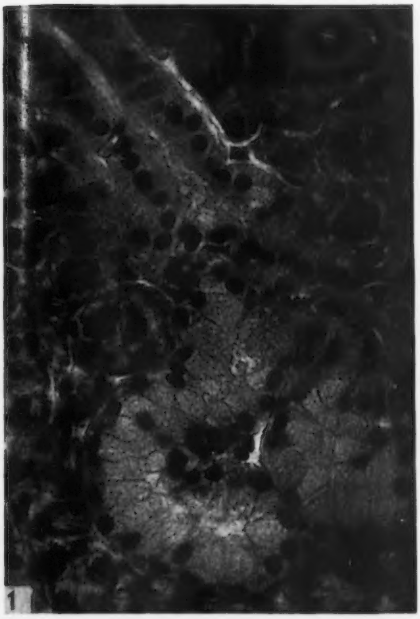
A serous tubule full of prominent granules of variable size is shown.

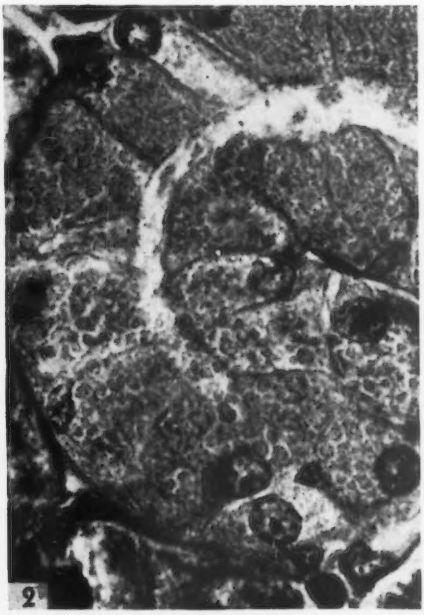
FIG. 3. Submaxillary gland of adult male rat fasted for 9 hours, then fed Purina fox chow for one hour, and killed immediately thereafter with chloroform. Susa fixation, H & E stain. ×500.

Granules are abundant in the serous tubules, although less than in those of Fig. 4.

FIG. 4. Submaxillary of adult male rat fasted for 9 hours, then fed Purina fox chow for one hour, and killed eight hours later. Susa fixation, H & E stain. ×500.

Note that there are somewhat more granules present in the serous tubules than in those of Fig. 3.





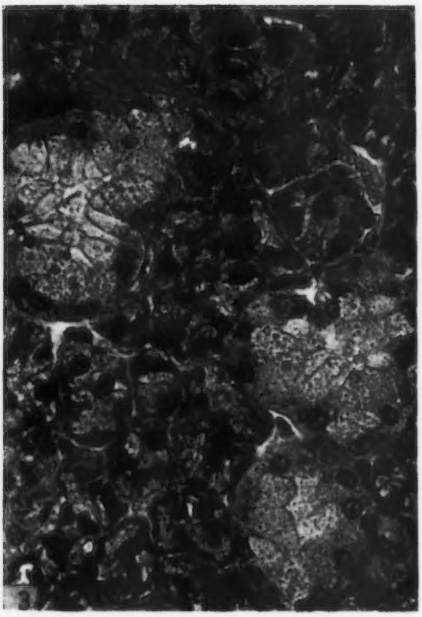


PLATE I

of the serous tubules were measured by ocular micrometer as indicated above and reported in Table 3.

MAIN EXPERIMENT

The joint action of testosterone and thyroxine was examined in an experi-

TABLE 3. THE EFFECT OF THYROIDECTOMY AND THYROXINE-TREATMENT ON THE SEROUS TUBULES OF THE SUBMAXILLARY GLAND OF THE RAT

	Average diameter of serous tubules (micra)	
	Males	Females
Normal Controls	45.8±0.48	42.3±0.35
Thyroidectomized	38.7±0.71	33.5±0.45
Thyroidectomized+Thyroxine	43.9±0.68	37.7±0.52

Statistical Analysis: All the differences between animals of different sexes receiving the same treatment, or of the same sex receiving different treatment were highly significant ($P<0.001$) except that the difference between the normal males and the thyroxine-treated thyroidectomized males was reliable to a probability of 0.05 and the difference between the latter group and the thyroidectomized males was reliable to a 0.01 probability.

TABLE 4. EFFECT OF TESTOSTERONE AND THYROXINE ON CASTRATED-THYROIDECTOMIZED RATS

		Control	Testosterone	Thyroxine	Testosterone and Thyroxine
Number of Animals at the End of the Experiment		8	6	7	8
Body Weight (gm.)	Initial	175	180	168	175
	Final	168	163	213	206
Oxygen Consumption (cc./hr./100 sq. cm.)	Initial	82	82	82	81
	Final	58	58	79	74
Heart Rate (beats per minute)	Initial	366	364	368	355
	Final	329	314	374	391
Seminal Vesicles	Weight (mg.)	58	856	66	754
Submaxillary and Sublingual	Weight (mg.)	350	366	436	536
	Relative weight (mg./100 gm. body wt.)	209	226	206	257
Kidney	Weight (mg.)	1030	1107	1548	1825
	Relative weight (mg./100 gm. body wt.)	613	681	730	890
Heart	Weight (mg.)	569	581	826	878
	Relative weight (mg./100 gm. body wt.)	340	348	392	429
Hypophysis	Weight (mg.)	12.9	8.1	9.7	6.6
	Relative weight (mg./100 gm. body wt.)	7.7	4.9	4.7	3.2
	Acidophiles	Absent	Absent	Normal	Normal
	Basophiles	Increased	Increased	Increased	Normal

Statistical Analysis of Relative Organ Weights: P Values

	Control vs. Testosterone	Control vs. Thyroxine	Control vs. Testosterone and Thyroxine	Testosterone vs. Thyroxine	Testosterone vs. Testosterone and Thyroxine	Thyroxine vs. Testosterone and Thyroxine
Submaxillary and Sublingual (mg./100 gm. body wt.)	0.298	0.800	0.001	0.261	0.162	0.010
Kidney (mg./100 gm. body wt.)	0.167	0.018	0.001	0.288	0.001	0.008
Heart (mg./100 gm. body wt.)	0.700	0.020	0.001	0.090	0.008	0.080
Hypophysis (mg./100 gm. body wt.)	0.001	0.001	0.001	0.700	0.009	0.009

ment including 4 groups of 10 young adult male rats each, all of which had been castrated and thyroidectomized. Starting from the day after operation, the animals were injected twice daily as follows: 1) A control group given 0.1 cc. of a saline solution containing 1% duponol C; 2) a testosterone group given 0.25 milligrams of free testosterone in 0.1 cc. of a saline solution containing 1% duponol C. Some heating was necessary to obtain a satisfactory suspension of testosterone in the duponol solution; 3) a thyroxine group given 3 micrograms of DL-sodium thyroxine in 0.1 cc. of water; 4) a testos-

TABLE 5. THE EFFECT OF TESTOSTERONE AND THYROXINE ON THE DIAMETER OF THE SEROUS TUBULES OF THE SUBMAXILLARY GLAND

			Control	Testosterone	Thyroxine	Testosterone and Thyroxine
Diameter of Serous Tubules (microns)			26.3	28.1	29.8	36.0
Per cent Increases of Tubular Width over Controls				7.1	13.3	37.2
Statistical Analysis: P Values						
	Control vs Testosterone	Control vs Thyroxine	Control vs Testosterone and Thyroxine	Testosterone vs Thyroxine	Testosterone vs Testosterone and Thyroxine	Thyroxine vs Testosterone and Thyroxine
Diameter of Serous Tubules (microns)	0.103	.009	.001	0.278	.001	.001

terone-thyroxine group receiving the 2 hormones at the doses indicated above, care being taken to inject testosterone on the one side of the body and thyroxine on the other throughout the experiment.

The animals were given a 1% solution of calcium lactate instead of drinking water in order to reduce the number of deaths from tetany. Their diet consisted of the following: corn meal, 70.9%; wheat gluten, 16.4%; dried brewer's yeast, 9.0%; pig liver, 1.9%; sodium chloride, 0.9% and calcium carbonate, 0.9%. It was found that this diet, which is a modification of Remington's iodine deficient diet No. 342 (Levine, Remington and von Kolnitz, 1933), made it possible to obtain thyroidectomized animals that did not gain weight and showed a complete absence of acidophilic cells in the hypophysis: complete absence of acidophiles in Susa fixed hypophyses of thyroidectomized rats is diagnostic of a successful thyroid ablation (Leblond, 1944). These results were not easily obtained with commercial feeds such as the Purina fox chow. On the other hand, thyroxine treatment produced a satisfactory growth in the castrated-thyroidectomized animals given the iodine deficient diet, as may be seen from the body weights in Table 4.

The animals were weighed at weekly intervals in the course of the experiment. Their oxygen consumption and heart rate were estimated by a method previously described (Leblond and Grad, 1948b).

The animals were sacrificed with chloroform 47 days after the hormone treatment had been instituted. A number of organs were fixed in Orth fluid,

weighed, sectioned and stained with hematoxylin-eosin. Table 4 includes the observed organ weights and the organ weights corrected for 100 grams of body weight. In the case of the hypophyses, which were fixed in Susa, the weights were reported along with the results of a microscopic search for acidophiles and thyroidectomy cells in the anterior lobe (Table 4).

The submaxillary glands, with the sublingual glands attached, were fixed in Orth, weighed and prepared like the other tissues. The diameters of the serous tubules were estimated as above (Table 5). A more precise histometric method was also used, in which the relative weights of the serous tubules and other parts of the glands were estimated from enlarged projections of the sections. The body tube of the microscope was placed in a horizontal position, 8″ above the table. A 45° projection prism (Bausch and Lomb) was adapted to the eyepiece to project the image on the table at a magnification of 430 times. For each animal, three fields were picked at random on the periphery of a section of the gland, and the outlines of the serous tubules and ducts were drawn on paper. The parts of the projection which did not represent either serous tubules or ducts consisted mostly of mucous acini with a few blood vessels, some connective tissue, and interstitial spaces. This was described as "remainder." Relative weights were estimated by cutting out of the paper the images of the various glands components and weighing them separately (Table 6). The percentage of the space occupied by each component was computed from these figures (Table 7).

The studies just reported were directed towards measuring any change in the size of the serous tubules under the different hormonal conditions. In the series of measurements now to be described, an attempt was made to determine whether the variation in amount of tubular tissue was accompanied by a change in the number of cells. To this end, the number of serous-tubule nuclei per field in 6 μ sections was counted for each animal, the average of which was reported in Table 8 as crude number of nuclei. The average diameter of the nuclei was also estimated and reported in the same table. Both measurements were performed on projections obtained as described above, but this time the magnification was 928. These data made it possible to calculate the true number of nuclei per field by Abercrombie's method (1946). On the assumption that the density of the tissue is equal to unity, the weight of the field could be estimated by multiplying its area by the thickness of the section. Knowing the number of nuclei per field, the weight of

EXPLANATION OF PLATE II

Submaxillary glands of 150 day old rats. The pictures on the left are from females; those on the right are from males. All photographs refer to Susa fixed, H & E stained sections. ×60.

FIG. 5. Intact female. The pale areas are the serous tubules.

FIG. 6. Intact male. The tubules are larger here than in females (Fig. 5).

FIG. 7. Thyroidectomized female. The tubules are smaller than those of normal females (Fig. 5).

FIG. 8. Thyroidectomized male. The tubules are smaller than those of normal males (Fig. 6), but somewhat larger than those of thyroidectomized females (Fig. 7).

FIG. 9. Thyroidectomized female treated with 15 micrograms of thyroxine daily for two weeks before autopsy. The tubules are larger than in untreated thyroidectomized females (Fig. 7).

FIG. 10. Thyroidectomized male treated with 15 micrograms of thyroxine daily for two weeks before autopsy. The tubules are larger than in untreated thyroidectomized males (Fig. 8).

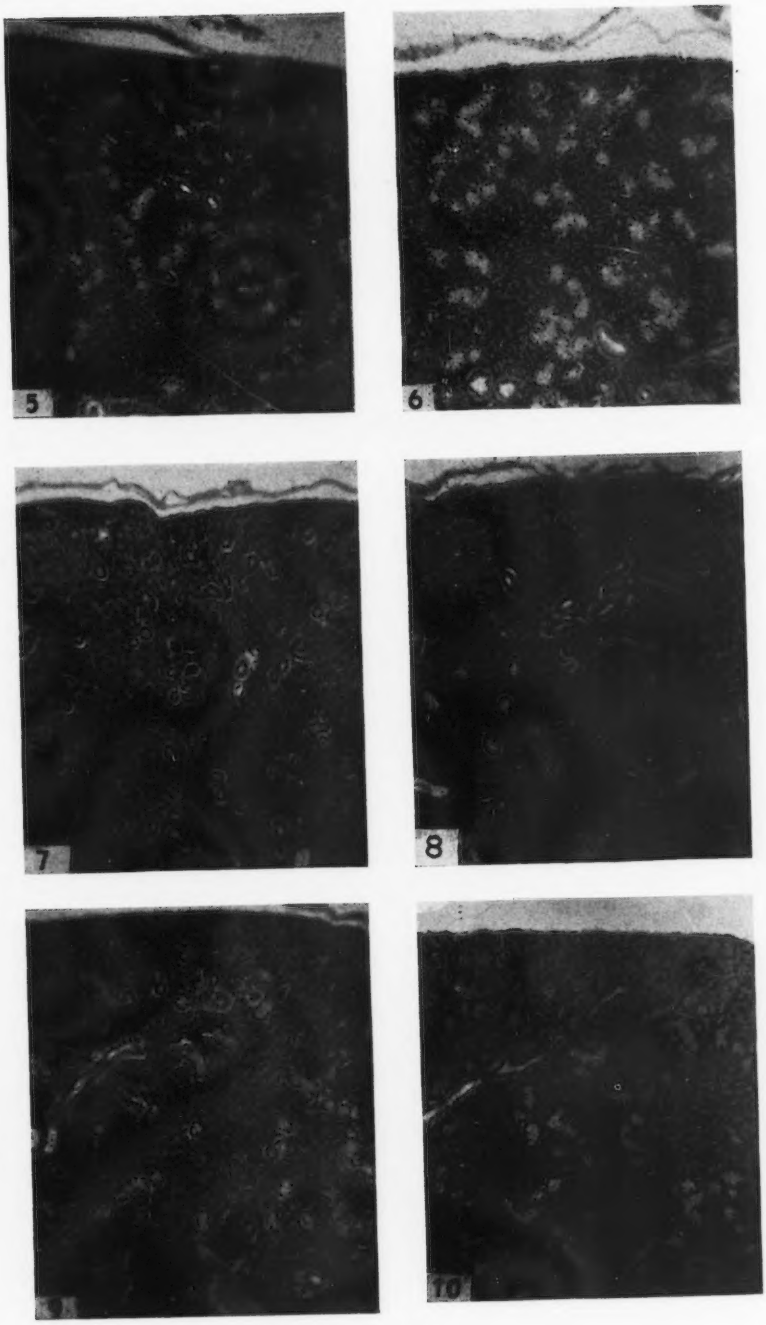


PLATE II

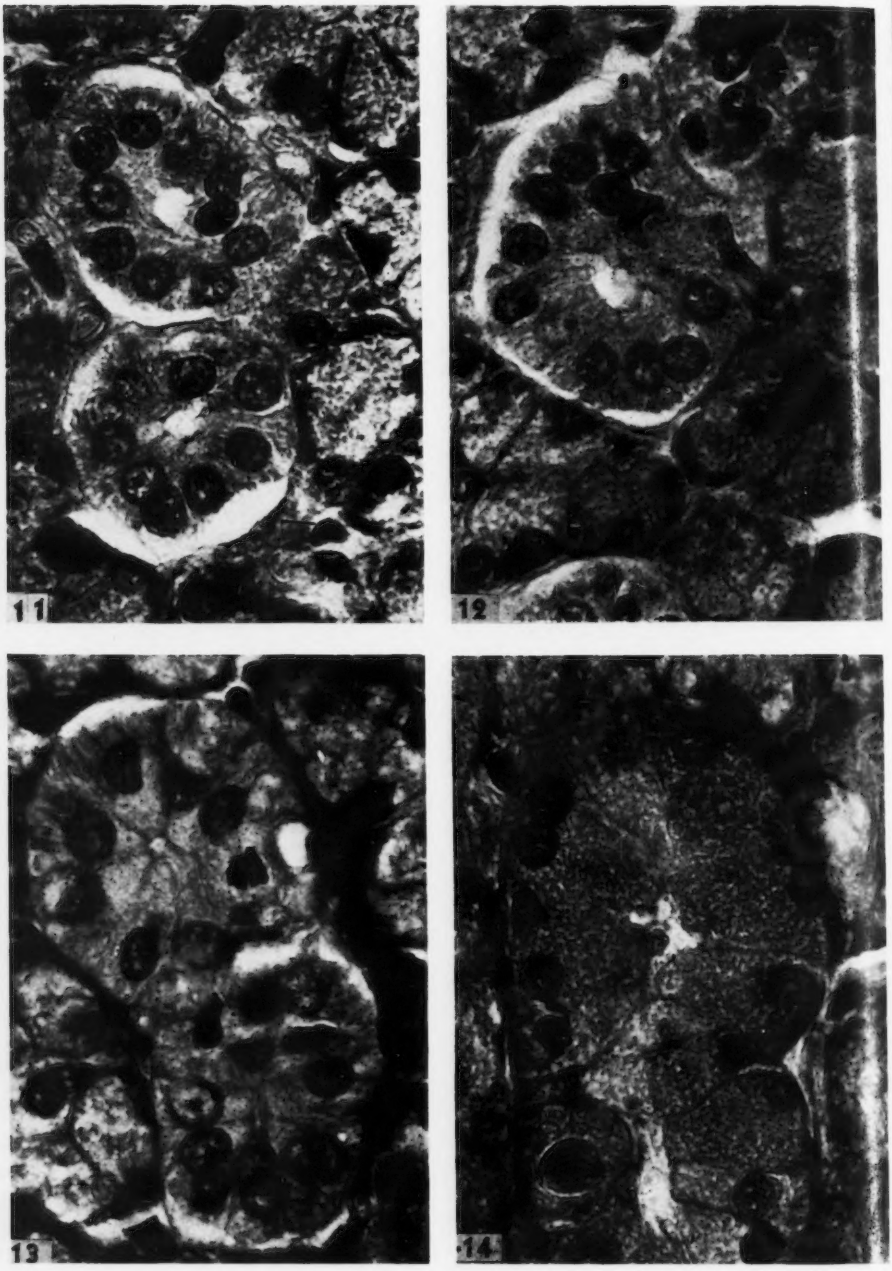


PLATE III

TABLE 6. THE EFFECT OF TESTOSTERONE AND THYROXINE ON THE RELATIVE WEIGHTS OF THE SUBMAXILLARY GLAND COMPONENTS, AS CALCULATED FROM PROJECTIONS OF MICROSCOPIC IMAGES

	Weights of Paper Projections (mg.)			
	Control	Testosterone	Thyroxine	Testosterone and Thyroxine
Serous Tubules	773.5	1071.6	1078.9	1301.6
Ducts	262.6	182.4	284.0	204.3
Remainder	4619.0	4316.6	4241.6	4154.3
Total	5655.1	5570.6	5604.5	5660.2

Statistical Analysis: P Values

	Control vs Testosterone	Control vs Thyroxine	Control vs Testosterone and Thyroxine	Testosterone vs Thyroxine	Testosterone vs Testosterone and Thyroxine	Thyroxine vs Testosterone and Thyroxine
Serous Tubules	0.158	0.097	0.009	0.676	0.255	0.252
Ducts	0.279	0.800	0.390	0.188	0.800	0.278
Remainder	0.100	0.047	0.019	0.646	0.378	0.700

TABLE 7. PERCENTAGE OF SUBMAXILLARY GLAND OCCUPIED BY SEROUS TUBULES, DUCTS, ETC., AS CALCULATED FROM MEASUREMENTS OF PROJECTIONS OF THE MICROSCOPIC IMAGES

	Control	Testosterone	Thyroxine	Testosterone and Thyroxine
Serous Tubules	13.7	19.1	19.3	23.0
Ducts	4.6	3.3	5.1	3.6
Remainder	81.7	77.6	75.6	73.4

each field, and the weight of the corresponding gland, it was possible to calculate the number of serous-tubule nuclei in the whole gland[2] (Table 8).

[2] The calculated number of nuclei was only an approximation, since the weight of the gland used in this calculation was the combined weight of the submaxillary and sublingual glands. However, the sublingual glands were considered small enough in regard to the submaxillary to justify neglecting them.

EXPLANATION OF PLATE III

Submaxillary glands from the main experiment. Orth fixation. H & E stain. ×1,100

FIG. 11. Control castrated-thyroidectomized male rat. Note the small size of the tubules and the complete lack of granules.

FIG. 12. Castrated-thyroidectomized male rat treated with testosterone. Note the slightly larger size of the tubules although granules are still not visible.

FIG. 13. Castrated-thyroidectomized male rat treated with thyroxine. The tubule is now larger than those in Figs. 11 and 12. Note also the mitotic figure. In common with the tubules of Figs. 11 and 12, there is still a deficiency of granules in the cells as well as a relatively central position of the nuclei.

FIG. 14. Castrated-thyroidectomized male rat treated with testosterone and thyroxine. Note the great increase in the width of the tubules over those of other groups, as well as the loading of the cytoplasm of the cells with granules which force the nuclei to a basal position. From a consideration of Figs. 11 to 14 inclusive, it is obvious that the tubules increase in size largely through the increase in the height of the cell and not through the increase of the lumen.

TABLE 8. THE EFFECT OF TESTOSTERONE AND THYROXINE ON THE NUMBER AND SIZE OF THE NUCLEI IN THE SEROUS TUBULES OF THE SUBMAXILLARY GLAND OF THE RAT

	Control	Testosterone	Thyroxine	Testosterone and Thyroxine
Crude Number of Nuclei per Field	83.9	77.7	82.5	68.5
True Number of Nuclei per Field	44.9	41.6	43.8	36.4
Diameter of Nucleus (micra)	5.2	5.1	5.3	5.3
Calculated Number of serous Nuclei in the Whole Gland ($\times 10^8$)	70.4	68.2	85.5	87.3

Statistical Analysis: P Values

	Control vs Testosterone	Control vs Thyroxine	Control vs Testosterone and Thyroxine	Testosterone vs Thyroxine	Testosterone vs Thyroxine and Testosterone	Thyroxine vs Thyroxine and Testosterone
Crude Number of Nuclei per Field	ca. 1.000	ca. 0.700	0.355	0.720	0.414	0.668
Diameter of Nucleus	ca. 0.600	ca. 0.565	ca. 0.600	0.350	0.363	ca. 1.000

In all experiments, standard errors were determined by analyses of variance, and significant differences found by "t" tests. According to the usual conventions, a P value lower than 0.05 was considered to be significant, while values lower than 0.01 were considered highly significant. Values lower than 0.001 were reported in the Tables as being equal to 0.001.

RESULTS

PRELIMINARY EXPERIMENTS

The effect of feeding and fasting on the serous tubules was only moderate. Histological examination revealed the presence of abundant granules in the serous cells of all groups. Some decrease in the amount of granules seemed to occur in the zero-hour group (Fig. 3), that is, at the end of a period of active feeding, while the number of granules appeared greater at 2 and 8 hours after the beginning of the fast (Fig. 4). However, measurements of the diameter of the serous tubules showed no significant differences between the zero-hour and the 2 or 8 hour groups (Table 1). The diameter of the tubules and the number of granules were definitely decreased in the group fasted for 120 hours (Table 1). It may be noted that in all groups, most of the ducts contained a rather large amount of secretion. No obvious differences in the nature and amount of duct content of the various groups could be observed.

The effect of age manifested itself by a gradual increase of the diameter of the serous tubules with increasing age. This increase was more pronounced between the 10th and 39th day, than between the 39th and 150th day (Table 2). Well developed secretion granules were present only in the oldest group.

The effect of sex was indicated by a greater diameter of the serous tubules in males than in females at 39 and 150 days. Similarly, in the next experiment (Table 3), males had larger tubules than females, whether the animals were intact, thyroidectomized or treated with thyroxine (Fig. 5–10). Such a result pointed to a role of the male hormone on the growth of the serous tubules.

The effect of thyroidectomy was also brought out by this experiment (Tables 3, Figs. 5–8), since in both sexes this operation reduced the size of the serous tubules. Furthermore, the tubules were partly restored by administration of thyroxine (Figs. 9–10). Histological examination under high power showed an almost complete absence of serous granules after thyroidectomy, while these were prominent in normal and thyroxine-treated animals. However, there was a possibility that thyroidectomy acted on the submaxillary glands by reducing the activity of the gonads and decreasing their secretion of testosterone. Another experiment was, therefore, necessary to separate the respective roles of thyroids and gonads.

The main experiment

The results of the main experiment (Table 4) were interpreted on the basis of an absence of hormonal secretion by testis and thyroid.

The effectiveness of castration was demonstrated by the atrophy of the seminal vesicles in the control and thyroxine treated groups. Conversely, the repair of castration symptoms by testosterone was demonstrated by the enlargement of the seminal vesicles in the two other groups.

The completeness of the thyroidectomy was demonstrated in the control and testosterone-treated group by the lack of gain in body weight, the decrease in oxygen consumption and heart rate, as well as by the complete absence of acidophiles and the presence of thyroidectomy cells in the hypophysis. Conversely, the action of thyroxine was demonstrated in the two remaining groups by the gain in body weight, the increase in oxygen consumption and heart rate, and the abundance of acidophiles in the hypophysis.

Testosterone alone had no statistically significant effect on the diameters (Table 5) and surface areas (Table 6 and 7) of the serous tubules. Thyroxine alone had a slight effect that was statistically significant only in the case of the diameters (Table 5). When both testosterone and thyroxine were administered jointly, there was a highly significant increase in both the diameters (Table 5) and surface areas (Table 6) of the serous tubules. It may be noted that both the diameters and the surface area were indices of cell size, since the lumina were small enough in all groups to be neglected (Figs. 11–14).

Microscopic examination revealed that the cells of the serous tubules in the operated control animals were shrunken and contained practically no granules (Fig. 11). In the animals treated with testosterone (Fig. 12) or thyroxine (Fig. 13), the cells appeared larger and

occasional granules were found. But the animals treated with both hormones had serous tubules distended with granules (Fig. 14), which pushed the nuclei against the basement membrane as in intact male rats.

The total number of nuclei in the serous tubules of the gland (Table 8) was not affected by testosterone alone, but was equally increased whether thyroxine was given alone or jointly with testosterone.

The joint action of thyroxine and testosterone was not confined to the submaxillary gland, but extended to the heart, kidney and hypophysis. In the case of heart and kidney, the moderate effect of testosterone alone was not statistically significant, but that of thyroxine alone was (Table 4). When testosterone was administered jointly with thyroxine, the increase in kidney weight became definitely greater than with thyroxine alone (Table 4), a fact already demonstrated by Masson and Romanchuck (1945). In fact, in the case of kidney and heart, the increase in weight due to the combined hormonal treatment was synergistic, that is to say, greater than the sum of the increases in weight due to each single hormonal treatment.

Testosterone and thyroxine together decreased the weight of the hypophysis more than either hormone alone (Table 4). Histological study of this gland revealed the presence of a typical thyroidectomy picture in the testosterone-treated group, since no acidophiles and many pale basophiles were found. A typical castration picture was noted in the thyroxine-treated group, since many acidophiles and more deeply stained basophiles were found. The animals given both hormones had normal-appearing hypophyses, while the untreated controls showed a combination of the signs of castration and thyroidectomy.

In contrast, the effect of testosterone on the seminal vesicles was less intense in the presence of thyroxine than in its absence, as shown by a significantly ($P = 0.008$) lower weight of the seminal vesicles after the combined hormonal treatment than after testosterone alone (Table 4). Masson and Romanchuck (1945) previously obtained the same results with daily doses of 60 micrograms of testosterone propionate and 10 to 40 micrograms of thyroxine. However, this effect was dependent on the dosage used, since later, Masson (1947) reported that 50 micrograms of testosterone propionate and 112.5 micrograms of thyroxine had a synergistic action on the weight of the seminal vesicles, thus confirming the earlier work of Caridroit and Arvy (1942).

DISCUSSION

Lacassagne showed that *testosterone* repairs the atrophy of the serous tubules brought about by castration in the male mouse (1940b). Frantz and Kirschbaum confirmed Lacassagne's results and

went so far as to consider the serous tubules as a more sensitive indicator of the presence of androgens than the seminal vesicles (1949a, b).

In an attempt to duplicate his results in rats, Lacassagne (1940c) concluded that the serous tubules in this species were also controlled by the male hormone. In agreement with this finding, the first results obtained in the present work showed a greater diameter of the tubules in male than in female rats, whether the animals were normal (Table 2), thyroidectomized or thyroidectomized and thyroxine treated

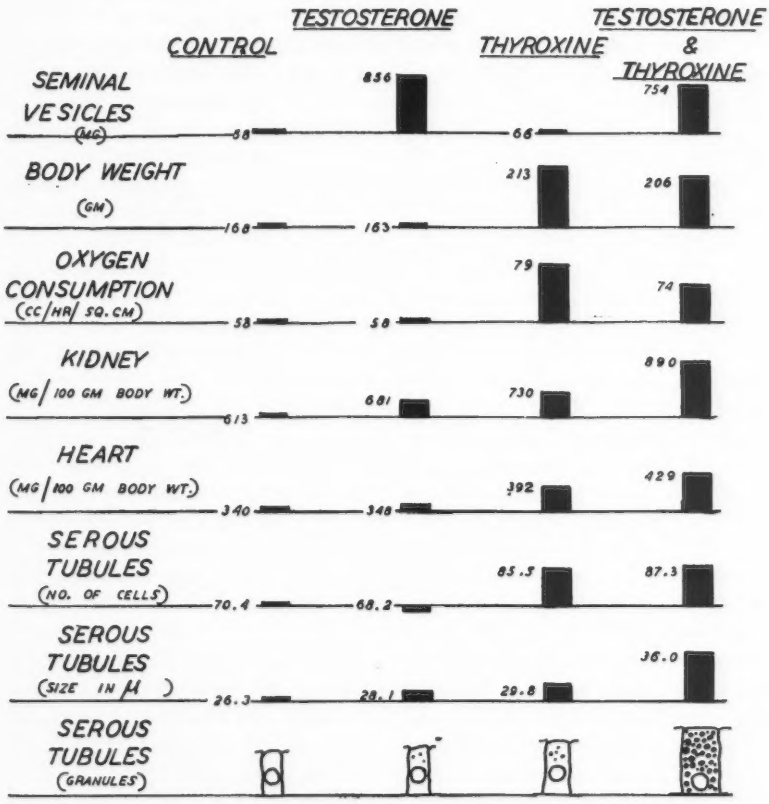


FIG. 15. Block diagram summarizing the effects of testosterone and/or thyroxine in castrated-thyroidectomized rats. In the case of each response the figure obtained for the controls was used as base line. The figures for seminal vesicle weight, body weight, oxygen consumption, kidney weight and heart weight were taken from Table 4. The figures for the number of cells in the serous tubules were taken from Table 8 and those for their size, from Table 5. Finally, a schematic drawing of the amount of granules in the cells of the serous tubules was added at the base of the chart. It is apparent that a synergistic action of testosterone and thyroxine is present only in the case of kidney weight, heart weight, and size and granule content of the serous tubules.

(Table 3). This sex difference had to be interpreted with caution, however, since the males, which had larger tubules than comparable females, also weighed more. Still, it suggested the possibility of an action of the male hormone on the serous tubules.

In the main experiment, in which the thyroid and testes of all animals had been removed, testosterone alone produced a large increase in the weight of the seminal vesicles, but had only a slight, statistically insignificant effect on the serous tubules (Fig. 15). In other words, testosterone had little or no action on the tubules, while its androgenic properties were fully evident. Therefore, the serous tubules did not behave as a secondary sex organ.

When *thyroxine* alone was administered, it raised the oxygen consumption to the maximum obtained in this experiment, but it caused only a slight increase in the size of the serous tubules (Fig. 15). This slight effect of thyroxine was significant when the criterion used was the diameter of the tubules (Table 5), but not when it was the surface area (Table 6). Furthermore, the serous granules were not restored to normal (Fig. 13). In contrast, thyroxine alone increased the number of serous cells to the maximum obtained in this experiment (Table 8). This response which paralleled that of oxygen consumption and body weight, seemed to be merely one more sign of the overall metabolic stimulation due to thyroxine.

While the two hormones given alone had little influence on the size and granule content of the serous tubules, the simultaneous administration of both *testosterone and thyroxine* produced a highly significant increase in the size of the tubules (Tables 5 and 6) and a massive accumulation of granules in their cells (Fig. 14). The combined effect of the hormones on the diameters of the tubules was synergistic, that is greater than the sum of the individual effects (Table 5). The formation of granules could also be considered as a synergistic response (Figs. 10–14). Of the end-organs listed in Fig. 15, only the kidney and heart weights showed a similar synergistic response to the two hormones.

Whether the participation of testosterone in the joint action on the serous tubules was exerted through its androgenic or other properties, and whether that of thyroxine was exerted through its metabolic-stimulating or other properties could not be definitely answered from this study.

While the results established the joint action of testosterone and thyroxine on the serous tubules of the castrated-thyroidectomized animals, the question arose as to whether a similar hormonal combination was responsible for the maintenance of these tubules in the normal male rat. In the first place, it could be assumed that testosterone is the hormone secreted by the rat testis (Moore, 1939), while thyroxine is secreted by the rat thyroid (Leblond and Gross, 1949). Secondly, the picture obtained by treatment of castrated-thyroidec-

tomized rats with these two hormones was similar to that found in the intact male rat. Therefore, it was concluded that under physiological conditions too, the joint action of these hormones may be held responsible for the maintenance of the serous tubules in the submaxillary gland of the rat.

In contrast to the profound influence of the testis and thyroid hormones on the serous tubules, the influence of *feeding and fasting* was surprisingly small (Table 1). The size of the tubules shrank significantly only after a 5-day fast. Even here hormones may have been involved, since a prolonged fasting was shown to decrease the activity of testis (Mulinos and Pomerantz, 1940) and thyroid (d'Angelo, Gordon and Charipper, 1949). Therefore, the direct influence of feeding and fasting—presumably exerted through the nervous supply of the gland—played no more than a minor role in maintaining the serous tubules.

The presence of granules in fed as well as in fasted animals, and the occurrence of secretion material in the lumen of the ducts in animals fasted for various lengths of time, suggested that some secretory activity of the gland was taking place more or less continuously, as already assumed by Babkin (1944) in the case of other digestive glands. Such a constant secretory activity might well be related to the continuous action of the testis and thyroid on the serous cells of the submaxillary gland.

SUMMARY

The object of this study was to examine the endocrine control of the serous tubules which, in addition to mucous acini, constitute the secretory portion of the submaxillary gland of the rat.

Preliminary experiments revealed that male rats had larger serous tubules than females (Table 2) and thyroidectomy decreased the size of the tubules in both sexes (Table 3). Therefore, both testis and thyroid seemed involved in the control of this structure. In order to assess the respective roles of these two glands, young adult male rats, which had been thyroidectomized and castrated, were treated daily for 47 days with 0.5 mg of testosterone or 6 micrograms of thryoxine or both, and the serous tubules were examined by histometric methods.

The results are as follows:

1) Control animals, in which testes and thyroids are extirpated, show an atrophy of the serous tubules.

2) Treatment of such animals with testosterone alone has little or no effect on the size and granule content of the cells of the serous tubules.

3) Treatment with thyroxine alone has little effect on the size and granule content of the cells of the serous tubules, but it restores the number of these cells to normal.

4) A combined treatment with both hormones restores a normal size and granule content as well as a normal number of the cells of the serous tubules (Figs. 11–14, Tables 5–8).

The testis and thyroid hormones in the doses indicated exert a synergistic action on kidney and heart as well as on the serous tubules of the submaxillary gland, but not on the male secondary sex organs, such as seminal vesicles, nor on the metabolic responses to thyroxine, such as the increase in oxygen consumption and body weight (Fig. 15).

In comparison with the major role of the testis and thyroid hormones in the maintenance of the serous tubules, nervous influences—such as those occurring in the course of feeding and fasting (Table 1)—have only a minor effect.

ACKNOWLEDGMENTS

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THE EFFECT OF GROWTH HORMONE UPON LIVER AND KIDNEY D-AMINO ACID OXIDASE AND UPON MUSCLE SUCCINIC ACID DEHYDROGENASE

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SINCE enzymes are proteins, and may indeed constitute a major part of the protein in cells (Sumner and Somers, 1947) several relationships between them and the growth hormone are conceivable. As catalysts, tissue enzymes might be chemical mediators through which certain effects of the hormone are produced; as proteins, they may simply participate in any increase of body protein. In the case of bone phosphatase, marked changes in concentration which are readily correlated with known effects of the growth hormone on skeletal development are observed after hypophysectomy and after replacement therapy, and a functional relationship is indicated (Mathies and Gaebler, 1949). The concentrations of the two enzymes discussed in this paper do not change during induced growth, so there is no indication of a relationship to the growth hormone differing from that of other tissue proteins.

EXPERIMENTAL

Intact rats of two strains and both sexes, 6 to 10 months old, and Sprague-Dawley female hypophysectomized rats, 1 to 2 months old, were used under conditions of *ad lib.* and paired feeding as indicated in the tables.

D-amino acid oxidase was determined essentially as outlined by Axelrod, *et al.* (1940). One ml. of 16.7% (1:5) liver homogenate or 0.5 to 1.0 ml. of 9.1% (1:10) kidney homogenate was placed in a 15 ml. Warburg vessel. One ml. of 0.1 M pyrophosphate buffer of pH 8.3 was added, plus distilled water to give a total volume of 2 ml.; 0.5 ml. of 2 M DL-alanine in the same pyrophosphate buffer was added from the side-arm after equilibration. Other conditions were: air in the gas space, 0.2 ml. of 10% KOH in the inset, and a digestion temperature of 38.0° C. Determinations were carried out in duplicate and blanks were run in every instance. The oxygen uptake is expressed as three times the 20 minute uptake after correction for the blank.

Succinic acid dehydrogenase was assayed as described by Umbriet, *et al.* (1945), except that 0.5 ml. water was omitted, giving a final

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TABLE 1. THE EFFECT OF GROWTH HORMONE ON D-AMINO ACID OXIDASE OF KIDNEY AND LIVER†

Experiment	No. of rats	Average body wt. at start of injection period in gm.	Average weight gained during injection period in gm.	Average terminal liver weight in gm.	Average terminal wt. of both kidneys in gm.	Average O_2 uptake in μl./hr.					
						Liver			Kidney		
						Per gm. tissue	Per mg. total N.	Per 100 gm.* body wt.	Per gm. tissue	Per mg. total N.	Per 100 gm.* body wt.
I. 8 month old Sprague-Dawley females, pair-fed. Given 3 mg. growth hormone (1485 R.U./gm.) per day/rat for 7 days.											
Treated	5	251	13	6.13	1.67	−258		− 600	−1387		− 876
Controls	5	253	5	6.64	1.62	−384		− 990	−1619		−1015
II. 6 month old "Edwards" females. *Ad lib.* fed. 6 mg. of above growth preparation given daily/rat for 6 days; 12 mg. on the 7th day.											
Treated	5	202	24	8.26	1.60	−565		−2060	−2370		−1680
Controls	5	208	1	7.34	1.53	−468		−1640	−1950		−1420
III. 8 month old Sprague-Dawley females, pair-fed. Given 6 mg. growth hormone (1740 R.U./gm.) per rat per day for 7 days; 12 mg. on the 8th day.											
Treated	5	256	21	8.44	1.91	−371	−10.28	−1131	−1850	−62.9	−1270
Controls	5	264	4	7.79	1.84	−242	− 6.80	− 704	−1780	−59.9	−1220
IV. 8 month old Sprague-Dawley females, pair-fed. Given 6 mg. growth hormone (1740 R.U./gm) per rat per day for 7 days.											
Treated	4	244	12	7.20	1.69	−402		−1130	−2070		−1370
Controls	5	257	1	7.57	1.77	−293		− 859	−1720		−1180
V. 8–10 month old Sprague-Dawley males. *Ad. lib.* fed; 10 mg. growth hormone (1740 R.U./gm.) given per day per rat for 14 days. Fasted 24 hrs. before sacrifice.											
Treated	5	481	16‡	10.96	2.79	−243	− 6.09	− 536	−1760	−56.0	− 988
Controls	5	471	4‡	11.13	2.79	−378	− 9.22	− 887	−2470	−77.2	−1450

* Calculated according to Miller (1948).
† Diet was Rockland rat diet (powdered for the paired-feeding experiments).
‡ Gain by the 13th day.

TABLE 2. TREATMENT OF HYPOPHYSECTOMIZED RATS WITH GROWTH HORMONE AND ITS EFFECT ON THEIR KIDNEY AND LIVER D-AMINO ACID OXIDASE

Experiment	No. of rats	Average body wt. at start of injection period in gm.	Average weight gained during injection period in gm.	Average terminal liver weight in gm.	Average terminal wt. of both kidneys in gm.	Average O_2 uptake in μl./hr.				
						Liver			Kidney	
						Per gm. tissue	Per mg. total N.	Per 100 gm. body wt.	Per gm. tissue	Per 100 gm. body wt.
I. 2 month old Sprague-Dawley female rats. Diet of Bennett, *et al.* (1948). Hypophysectomized rats fed *ad lib.*; controls pair-fed against them. Plateaued for 14 days after operation and then injected 1 mg. growth preparation, daily per rat for 17 days (1384 R.U./gm.). B1 fed against A1; B2 against A2.										
A. Hypophysectomized.										
1. Treated	6	154	53	6.80	1.00	−132	−3.98	−432	−2380	−1150
2. Controls	5	146	−1	4.65	0.75	−162	−4.46	−518	−2270	−1170
B. Normal.										
1. Treated	5	175	25	5.45	1.11	−234	−6.14	−639	−2330	−1297
2. Controls	5	165	−1	4.27	1.00	−200	−5.17	−518	−2220	−1350
II. *Same as above, except a 24-day injection period was used and all rats were pair-fed against group A2.										
A. Hypophysectomized.										
1. Treated	5	176	46	5.39	1.10	−135	−3.93	−326		
2. Controls	7	173	5	5.80	0.88	−135	−3.69	−430		
B. Normal.										
1. Treated	5	189	23	4.86	1.24	−234	−6.07	−536		
2. Controls	6	191	2	4.62	1.34	−234	−6.35	−560		

* Enzyme assays carried out on pooled samples.

volume of 2.5 ml. The succinate was added from a side-arm at zero time as recommended by Axelrod, *et al.* (1942) for muscle, and a single concentration of muscle (0.2 ml. of 16.7% or 1:5) was employed in the duplicates while 2 concentrations of cytochrome c (0.2 ml. and 0.3 ml. of the stock solution, 0.1 ml. being the amount at which O_2 uptakes plateaued) were used. Blanks were found to be unnecessary. The oxygen uptake is expressed as two times the 30 minute uptake.

The kidneys, liver, and muscle (tibialis anticus) were removed immediately after sacrifice and weighed. Tissues were analyzed on the day of sacrifice in most instances; those which were not immediately assayed were stored at −18° C. Homogenates were prepared in the usual manner and assayed immediately after preparation. Muscle was first disintegrated by freezing with liquid air in a steel mortar and pulverizing by a sharp blow on the pestle, before homogenizing.

Micro-Kjeldahl determinations were carried out according to Hiller, Plazin and Van Slyke (1948).

RESULTS

1. D-amino Acid Oxidase. As mentioned in a preliminary communication (Mathies, 1949) *in vitro* experiments gave no indication of any specific relationship between growth hormone and D-amino acid oxidase. Experiments summarized in Table 1 were carried out on mature white rats to determine whether an *in vivo* effect could be demonstrated. An hereditary difference may exist, for the "Edwards" animals have a greater oxidase content than the Sprague-Dawley strain. No significant sex difference is apparent. Within wide limits, individual variations in the content of this enzyme being high, the concentration of oxidase in the liver appears to parallel that of the kidney, averaging one fifth of the kidney concentration. Also, the total amount in the liver is approximately equal to that in the kidney. This would seem to be true for the mature animal, but not the immature animal, as lower liver values were obtained in the latter. There appears to be no significant alteration in the enzyme after injections of growth preparation. The concentration remained essentially unchanged, and it would seem that the enzyme was synthesized in proportion to the growth in tissue.

To demonstrate this more conclusively, larger weight changes were required. The growth response of immature hypophysectomized female rats was therefore investigated. Results in Table 2 indicate that no change in the concentration of the enzyme occurred in spite of the high rate of induced growth. Group IA showed an increase in kidney weight of 33%, while the concentration of oxidase remained the same. Evidently, the added tissue contained its normal complement of the enzyme. Liver weight responded similarly, and it is evident that oxidase was also synthesized here at a rate proportional to growth. Normal animals, treated and untreated (Table 2, groups

IB and IIB), have a higher concentration of the enzyme in the liver than treated and untreated hypophysectomized animals. This is probably related to the loss of the thyrotropic hormone secretion of the anterior hypophysis, since Klein (1939) demonstrated that thyroidectomy lowered, and hyperthyroidism increased, liver D-amino acid oxidase.

2. Succinic Acid Dehydrogenase. Muscle succinic acid dehydrogenase was similarly investigated in two series of animals. Group IA1 (Table 3) shows an increase of 46% in muscle weight on treatment. Compared with changes of +19% in tibial weight, +25% in body weight, −7% in liver weight and +25% in kidney weight, it is apparent that the great bulk of growth was in the musculature, pro-

TABLE 3. MUSCLE SUCCINIC ACID DEHYDROGENASE

Experiment	No. of rats	Average wt. gain in gm.	Average terminal muscle weight in gm.	Average muscle % N.	Per cent increase in muscle wt. by treatment	Average O_2 uptake in μl./h		
						Per gm. tissue	Per mg. total N.	Per 100 gm. body wt.
I. Same group as II in Table 2.								
A. Hypophysectomized.								
1. Treated	5	46	0.469		+46	−4860		−1028
2. Controls	7	5	0.321			−5520		− 995
B. Normal								
1. Treated	5	23	0.455		+ 9	−5400		−1160
2. Controls	6	2	0.417			−5880		−1270
II. 1 month old Sprague-Dawley female rats. Diet of Bartlett (1949). All rats hypophysectomized at 1 month. Plateaued 10 days. Injected with 0.05 mg. growth hormone (1361 R.U./gm.)/rat/day for 6 days, then 0.1 mg./rat/day for 5 days and sacrificed.								
A. Treated	10	14.1	0.174	3.54	+25	−8400*	−237	
B. Controls	10	0.1	0.139	3.45		−8100*	−235	

* Pooled muscle samples assayed.

vided that results for one muscle can be extrapolated to include all muscle. Calculations based on this assumption indicate that 80% of the growth occurred in the muscle, a result in agreement with that of other investigators (Lee and Schaffer, 1934). With this in mind, it is extremely interesting to note that the enzyme concentration was maintained at or near normal values. Thus, 46% of the enzyme present was synthesized as a direct result of growth hormone treatment. In this respect, normal conditions for aerobic oxidation are maintained in this rapidly formed muscle. In view of the known glycostatic effect of growth hormone (Wilhelmi, Fishman, and Russell, 1948), this fact assumes added importance.

A lower level of growth hormone treatment (Table 3, group II) gave increases of 22% in tibial weight, 25% in muscle weight, and 15% in body weight. The muscle response is again about twice that of the total weight gain, but the tibial weight response is even higher than in the first experiment, in which the treatment period was longer and amount of growth hormone given was larger. There are various possible explanations for this difference. The ages were

different, different hormone preparations were used, and tibial weight might have a higher initial growth rate plateauing sooner.

SUMMARY

The effect of growth hormone on kidney and liver D-amino acid oxidase, muscle succinic acid dehydrogenase, and kidney, liver and muscle wet weight was investigated. The concentrations of the two enzymes in their respective tissues remained constant in spite of extensive tissue growth, indicating synthesis of these enzymes in direct proportion to tissue growth. Hypophysectomy resulted in reduced liver D-amino acid oxidase concentrations, probably as a result of reduced thyroid activity.

ACKNOWLEDGMENTS

We wish to express our thanks to Dr. P. D. Bartlett for tissues assayed in Table 3, group II. The growth hormone preparations used in this study were obtained from Parke, Davis & Co. in generous supply through the kindness of Dr. D. A. McGinty and Mr. L. W. Donaldson. The assays had been made by the "plateaued" female rat technique.

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THE EFFECTS OF PITRESSIN AND DESOXYCORTICOSTERONE IN LOW DOSAGE ON THE EXCRETION OF SODIUM, POTASSIUM, AND WATER BY THE NORMAL DOG[1]

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In 1938, Silvette and Britton presented evidence that there exists a physiological antagonism between the diuretic action of adrenal cortical hormone and the antidiuretic action of posterior pituitary hormone. An antagonism between the sodium and chloride conserving effects of the former and the chloruretic and natriuretic effects of the latter has also been postulated—first by Silvette and Britton (1938), and more recently by Roemmelt, Sartorius and Pitts (1949). It has been suggested that the major disturbances in electrolyte and water metabolism of the adrenalectomized animal resemble an exaggerated effect of posterior pituitary antidiuretic hormone (Roemmelt *et al.*, 1949, Birnie *et al.*, 1949). As desoxycorticosterone is known to correct the disturbed electrolyte balance in Addison's disease without significantly altering the disturbed water balance (Talbott *et al.*, 1942; Reforzo-Membrives *et al.*, 1945), it seemed reasonable to predict that desoxycorticosterone would reduce the natriuresis but have no effect on the antidiuresis produced by the administration of pitressin to a normal animal. The data presented in this paper bear out these inferences.

METHODS

Two types of experiments were performed on normal unanesthetized female mongrel dogs weighing from 12 to 18 kilos. In the first type of experiment the dogs were infused with creatinine in 0.45 per cent saline at a rate of 1 cc. per min. in order to determine glomerular filtration rates and the time courses of action of pitressin and desoxycorticosterone. In a second type of experiment the dogs were given a 40 cc. per kilo water load following two 15 minute control periods. In this type of experiment changes in urine flow and sodium excretion were of greater magnitude and more reproducible. We have therefore employed the water loading procedure in an evaluation of the effects of small doses of desoxycorticosterone and pitressin. In order to obtain uniform results it was necessary to standardize procedures in such a

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way as to ensure control urine flows within limits of .25 and .75 cc's per minute and control rates of sodium excretion within limits of 10 and 30 microequivalents per minute. Crystalline desoxycorticosterone acetate[2] dissolved in sesame oil and homogenized with water was given intravenously at the same time as the water load. Due to the delayed action of desoxycorticosterone, pitressin was given intravenously 15 minutes later. Sodium and potassium were determined by an internal standard flame photometer with an accuracy within $\pm 2\%$. Creatinine determination and other techniques have been described in a previous communication (Sartorius *et al.*, 1949).

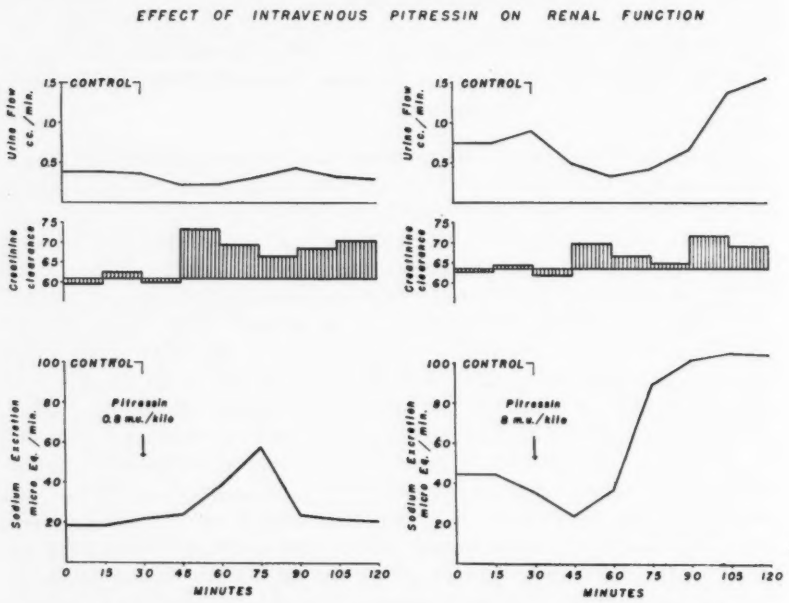

FIG. 1. Immediate effects of intravenous administration of 0.8 and 8.0 m.u. per kilo of pitressin on urine flow, filtration rate and sodium excretion of the normal dog.

RESULTS

Effects of Intravenous Pitressin on Renal Function

In a series of experiments performed on normal, well hydrated dogs, the antidiuretic and natriuretic effects of single intravenous injections of pitressin in dosage from 0.4 m.u. per kilo to 10 m.u. per kilo were determined. Two representative experiments performed on a single animal in which 0.8 m.u. and 8.0 m.u. per kilo of pitressin were employed are shown in figure 1. On the left side of the figure are shown the effects of 0.8 m.u. per kilo of pitressin on urine flow, glomerular

[2] We are indebted to Ciba Pharmaceutical Products, Inc. for the generous supply of desoxycorticosterone used in these experiments.

filtration rate and sodium excretion, while on the right side the effects of 10 times the dose is charted. In each experiment two 15 minute control periods preceded the pitressin. Following the injection of pitressin no significant change occurred in glomerular filtration rate for the first 15 minutes. However, in the subsequent 15 minute period filtration rate rose sharply in all experiments and then either gradually decreased or remained essentially constant for the remainder of the experiment. No direct correlation was found between the dosage of

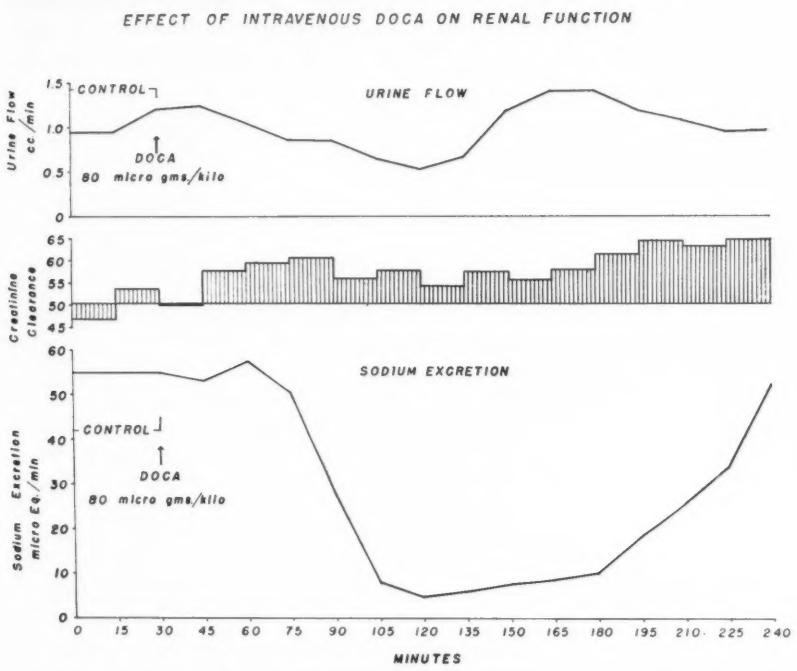

FIG. 2. Immediate effects of intravenous administration of 80 microgms./kilo of desoxycorticosterone on urine flow, filtration rare and sodium excretion of the normal dog.

pitressin and the rise in creatinine clearance. Urine flow invariably decreased immediately following the injection of pitressin reaching a minimum approximately 30 minutes later. Sodium excretion remained unchanged for 15 minutes following the injection. With 0.8 m.u. per kilo, a rise in sodium excretion occurred during the next 30 minutes so that peak excretion was attained approximately 45 minutes following the pitressin. A rapid fall was then commonly observed. As may be seen from the right side of figure 1 when larger doses of pitressin were given the natriuretic effect was more sustained and of greater magnitude although the same latency was observed. It is

interesting to note that with larger dosage of pitressin an increase in urine flow commonly occurred one to 1½ hours after pitressin, on occasion reaching 4–5 cc. per minute. The natriuresis always preceded a pitressin diuresis in these experiments.

Effects of Intravenous Desoxycorticosterone Acetate on Renal Function

The effects of intravenous desoxycorticosterone on renal function in a representative experiment are shown in figure 2. Following two 15 minute control periods, 80 micrograms per kilo of desoxycorticosterone in 1 cc. volume were administered intravenously. As with pitressin a fairly sharp rise in glomerular filtration rate was observed,

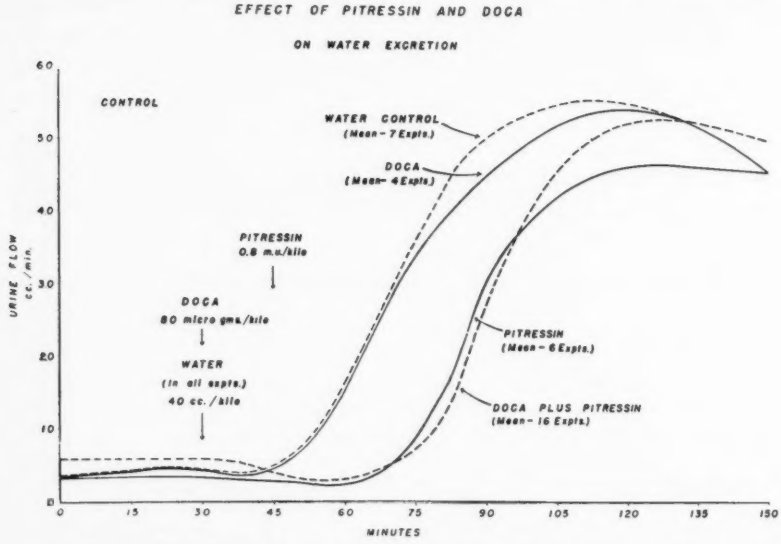

FIG. 3. The effect of pitressin and desoxycorticosterone on excretion of a standard water load by the normal dog.

this elevation being maintained throughout the 4 hour period of the experiment. Urine flow decreased slightly, reaching a minimum at the time of maximum sodium reabsorption. Following the period of maximum sodium reabsorption, there usually occurred a moderate diuresis, often resulting in a total net fluid loss. In the lower half of the chart the effects of desoxycorticosterone on sodium excretion are plotted as a function of time. Following a latent period of approximately 45 minutes sodium excretion decreased rapidly, from an initial level of 65 microequiv. per minute to a low of 5 microequiv. per minute. Diminished sodium excretion was maintained for approximately 75 minutes following which there occurred a gradual return to the control level.

Combined Effects of Pitressin and Desoxycorticosterone on Water Excretion

The data presented in figure 3 provide the basis for our conclusion that desoxycorticosterone has no effect on the elimination of water following a water load nor does it antagonize the antidiuretic action of pitressin. The several curves plotted on this figure are averages of the numbers of experiments noted. For a better evaluation of the combined effects of small doses of desoxycorticosterone and pitressin on water excretion, we have employed the water loading procedure described above. In all experiments therefore, a standard

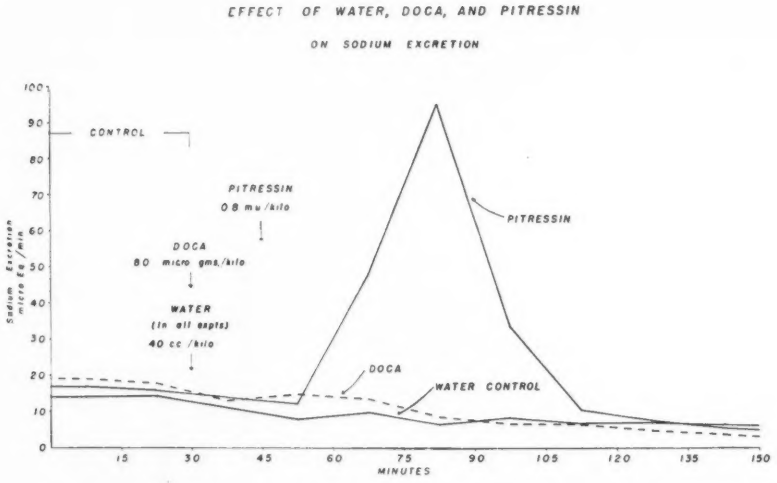

FIG. 4. The effect of pitressin and desoxycorticosterone on sodium excretion by the normal dog under the influence of a standard water load.

water load of 40 cc/kg was administered per os following two 15 minute control periods. In the dashed water control curve no other variable was introduced. This curve therefore constitutes a control for all other procedures. The solid curve adjacent to it and labeled DOCA, differs only in the fact that 80 micrograms per kilo of desoxycorticosterone or 1 to 1½ mg. as a total dose were administered at the same time as the water load. Differences between these curves have no significance. The continuous line labeled pitressin represents the mean urine flow of dogs receiving 0.8 m.u. per kilo of pitressin given 15 minutes after the water load. A marked reduction of output during the subsequent 45 minutes is apparent. At 45 minutes after the water load the average urine flow of the dogs receiving pitressin was 0.9 cc. per minute as compared to 3.6 cc. per minute for the controls. The lower interrupted line labeled DOCA plus pitressin represents the mean urine flow of the dogs receiving the same dose of pitressin but

in addition desoxycorticosterone in dosage varying from 0.8 micrograms per kilo to 80 micrograms per kilo. Again it is evident that desoxycorticosterone exerted no significant effect on water excretion, that is, it does not antagonize the antidiuresis produced by pitressin.

Combined Effects of Pitressin and Desoxycorticosterone on Sodium Excretion

Figure 4 portrays the natriuretic action of pitressin in the water loading experiments just outlined and compares this action to the sodium conserving effect of desoxycorticosterone and of even the water load by itself. In the lowermost curve labeled water control it is apparent that a water load of 40 cc. per kilo, uncomplicated by other procedures diminished sodium excretion gradually from an

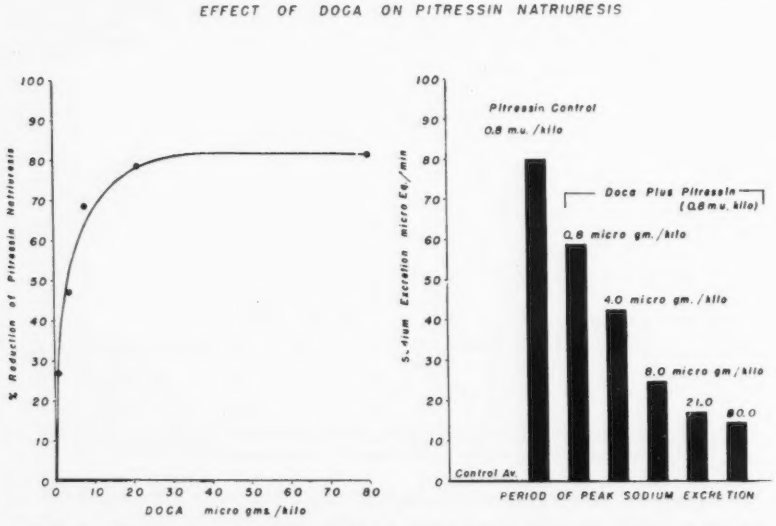

FIG. 5. The antagonistic action of desoxycorticosterone on increased sodium excretion produced by the intravenous administration of 0.8 m.u. per kilo of pitressin to the normal dog.

initial level of 14 microequiv. per min. to 6 microequiv. per min. two hours later. In other experiments of longer duration it has been observed that sodium excretion returned to the control level approximately 3 hours following the water load. Charted as an interrupted line, desoxycorticosterone in dosage of 80 micrograms per kilo reduced sodium excretion from a control level of 18 microequiv. per min. to a low of 3 microequiv. per min. two hours later. Pitressin in dosage of 0.8 m.u. per kilo or 10 to 15 m.u. per dog caused an increased sodium excretion of 80 microequiv. per min. attaining a peak excretion 45 minutes following the injection. There was a latent period of 15

minutes before any natriuretic effect was apparent. Seventy-five minutes following the injection of pitressin sodium excretion again returned to control or sub-control levels.

The effects of desoxycorticosterone on pitressin natriuresis are presented in figure 5. On the right side of the chart, sodium excretion in microequiv. per min. above a control level is shown for the period of peak pitressin natriuresis. The first column represents the mean natriuretic effect of 6 experiments in which 0.8 m.u. per kilo of pitressin was given 15 minutes after the water load. On an average, this dose of pitressin caused the excretion of an extra 80 microequivalents of sodium during its peak period of action. In each of the other 5 columns this same amount of pitressin was given but in addition desoxycorticosterone was administered in the dosage indicated. When 0.8 microgms/kilo of desoxycorticosterone was given, sodium excretion rose to 59 microequiv. per min., a reduction of 21 microequivalents when compared to pitressin alone. Increasing dosage of desoxycorticosterone decreased pitressin natriuresis progressively in stepwise fashion until in the last column when 80 microgms/kilo were given, sodium excretion rose only 14 microequiv. per min. above the base control level.

When these results are plotted as per cent reduction in pitressin natriuresis against desoxycorticosterone dosage the curve shown on the left hand side of figure 5 is obtained. It would thus appear that desoxycorticosterone in any dosage is unable to counteract completely the natriuretic effect of 0.8 m.u. per kilo of pitressin. From other experiments which have not as yet been completed in their entirety it would appear that with smaller doses of pitressin this limitation is negligible, while with larger doses it becomes even more apparent.

Combined Effects of Pitressin and Desoxycorticosterone on Potassium Excretion

Our data on potassium excretion, figure 6, is derived from but ten of the 33 experiments reported above, since the number of experiments suitable for a presentation of average values is limited to those with comparable control potassium excretion rates. However, potassium excretion in all experiments, regardless of control values, showed a similar trend but of varying magnitude. In the lowermost curve of figure 6 and labeled water control, it is apparent that a water load of 40 cc./kilo reduced potassium excretion from an initial level of 21 microequiv. per min. to a low of 8 microequiv. per min. Both desoxycorticosterone in dosage of 80 microgms. per kilo and pitressin in dosage of 0.8 m.u. per kilo increased potassium excretion an average of approximately 13 microequiv. per min. A latent period of 15 minutes preceded the increased rate of potassium excretion due to the administration of desoxycorticosterone. No latent period was apparent after pitressin injection. The combined actions of pitressin and

desoxycorticosterone in the dosage indicated above is graphed as a dash-dot line in figure 6. Almost complete summation is evident.

DISCUSSION

The low volume and high salt content of the urine of adrenalectomized animals and of patients with Addison's disease suggest an exaggerated effect of, or an actual increase in the amount of circulat-

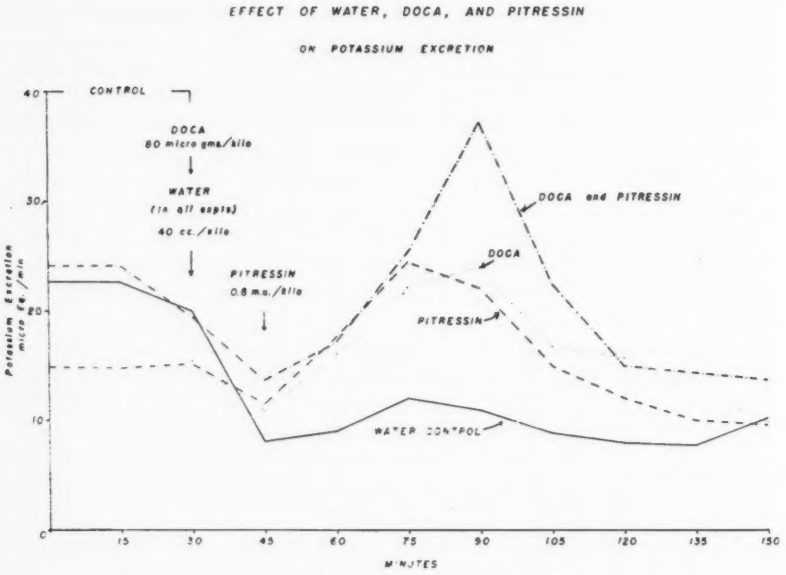

FIG. 6. The synergistic effect of pitressin and desoxycorticosterone administration to the normal dog on potassium excretion.

ing posterior pituitary antidiuretic hormone (Roemmelt *et al.*, 1949, Birnie *et al.*, 1949). This hypothesis recognizes the antidiuretic, chloruretic and natriuretic actions of the posterior pituitary hormone as being in physiological balance with the diuretic and salt conserving actions of the cortical hormones (Silvette and Britton, 1938, Corey, Silvette and Britton, 1939 and Winter *et al.* 1939). Assuming the above concept to be true, it seemed logical that the administration of pitressin in small dosage to a normal animal would produce a mild pseudo-Addisonian state in respect to electrolyte and water balance and that such disturbances would be amenable to desoxycorticosterone administration to the same extent as are those of the adrenalectomized animal.

That pitressin inhibits water diuresis is well known (Smith, 1947). That desoxycorticosterone exhibits diuretic properties has been

suggested by numerous investigators (Selye and Basset, 1940; Corey and Britton, 1941; Eversole *et al.* 1942). As the data in the experiments pertaining to the diuretic activity of desoxycorticosterone were derived from 11, 12 or 24 hour urine collection periods, the *modus operandi* of this action has not been clarified. Ragan *et al.* 1940 investigated the diuretic action of desoxycorticosterone by producing in dogs, a diabetes insipidus like state through the administration of 25 mg. of the drug daily. They arrived at the conclusion as did Mulino, 1941 that this condition was due primarily to increased thirst of the animals and that the polyuria was a secondary manifestation. This concept is in accord with our findings that the intravenous administration of desoxycorticosterone results primarily in increased salt reabsorption which is attended by increased water absorption of lesser magnitude, as shown in figure 2. Any subsequent diuresis then would be secondary to expanded blood volume as a consequence of desoxycorticosterone administration (Swingle and Remington, 1944), to elevation of filtration rate (Winter and Ingrams, 1943), or to increased water intake dependent upon increased sodium reabsorption (Anderson and Marlin, 1942). It has also been shown that the deficiencies in handling water of 6th day post adrenalectomized rats can be only partially corrected by the administration of either desoxycorticosterone or whole cortical extract (Gaunt, 1946). Patients with Addison's disease show a similar refractoriness to correction of water balance (Reforzo-Membrives, 1945, and Talbott, 1942). Since pitressin exerts a specific action on the capacity of the renal tubule to reabsorb water (Smith, 1947) and since desoxycorticosterone has been shown in the above experiments to exert neither a direct action on water reabsorption per se, nor an antagonistic action to pitressin antidiuresis, clarity is added to our understanding of the adrenalectomized animal's relative incapacity to excrete water under desoxycorticosterone therapy. Whether some fraction of the adrenal cortex possesses direct diuretic activity (Silvette and Britton, 1938) or whether the intra-renal circulation undergoes alteration as a result of the exaggerated effects of antidiuretic hormone (Trueta *et al.* 1947) thus accounting for the progressive refractoriness of the adrenalectomized rat's ability to excrete water under therapy (Gaunt, 1946) is not clear at the present time.

The natriuretic action of pitressin has been previously demonstrated (Anslow *et al.*, 1948 and Roemmelt, 1949) and has, in the present experiments, been found to be of fairly constant magnitude and extent. Desoxycorticosterone in low dosage progressively but never completely counteracted the natriuretic activity of 0.8 m.u. per kilo of pitressin as shown in figure 6. It seems probable that with lower pitressin dosage, the antagonistic action of desoxycorticosterone on sodium absorption would be complete. We would also suspect from the above findings that the physiological dose of pitressin lies

somewhat below a level of 0.8 m.u. per kilo. The efficacy of desoxycorticosterone therapy in correcting and maintaining proper sodium excretion in adrenalectomized animals and in Addisonian patients has been adequately demonstrated (Thorn, 1938, Britton and Kline, 1941).

Potassium excretion is diminished in the adrenalectomized animal (Harrop, 1933) and can be increased by the administration of desoxycorticosterone (Wells, 1940 and Swingle, 1944). Our experiments indicate that pitressin in low dosage increases the excretion of potassium. This increase is independent of the hyperkalemia which results from the injection of fairly large doses of pitressin (Yanagi, 1938) and likewise bears no relationship to the increase in filtration rate often observed following the injection of 0.8 m.u. per kilo of pitressin. This observation at first glance does not appear compatible with a concept of adrenal and posterior pituitary hormonal antagonism. However, since the independent actions of pitressin and desoxycorticosterone showed almost complete summation of the excess potassium excreted when used in combination, it becomes apparent that the adrenalectomized animal should, and indeed does, show a diminished rate of potassium excretion. It is likewise evident that the administration of pitressin to a normal animal cannot reproduce in its entirety the Addisonian electrolyte and water imbalances, since the combined effect of pitressin and cortical hormone on potassium excretion is additive rather than antagonistic.

In conclusion, it appears that desoxycorticosterone is antagonistic to the posterior pituitary antidiuretic hormone as to sodium excretion, synergistic as to potassium excretion, and without direct effect as to water excretion.

SUMMARY

The effects of intravenous injection of desoxycorticosterone and pitressin were studied as to filtration rate, sodium excretion and urine flow of the normal dog. The time courses of action of these drugs were determined.

In a second type of experiment, water excretion curves were established for dogs given 40 cc./kilo water load following two 15 minute control periods. Pitressin in dosage of 0.8 m.u. per kilo given 15 minutes following the water load exerted an antidiuretic effect of $1\frac{1}{2}$ to 2 hours duration. Desoxycorticosterone in dosage from 0.8 micrograms per kilo to 80 micrograms per kilo exerted no effect on either water diuresis or on the antidiuretic action of pitressin.

Eight-tenths micrograms per kilo of pitressin caused the excretion of an extra 80 microequiv. per min. of sodium during its peak period of action. Increasing dosage of desoxycorticosterone decreased the pitressin natriuresis but did not completely block this effect.

Both desoxycorticosterone and pitressin independently increased

the rate of potassium excretion and when given in combination showed almost complete summation of the excess potassium excreted.

The interrelationships and antagonistic actions of the posterior pituitary antidiuretic hormone and the adrenal cortical hormones are discussed.

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FACTORS INFLUENCING LOBULO-ALVEOLAR DEVELOPMENT AND MAMMARY SECRETION IN THE RAT[1]

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DURING pregnancy, the mammary gland of the rat undergoes a marked transformation so that, near the end of gestation, it consists of grape-like clusters of alveoli in lobule formation. Two days before parturition, secretion within the alveoli becomes visible (Turner and Schultze, 1931). Full development of the mammary gland, therefore, is attained when lobulo-alveolar development with secretion is established. In the absence of pregnancy, full development of the mammary gland has been produced experimentally in rats by injection of the gonadotropic substance from the urine of pregnant women (Selye *et al.*, 1933). This hormone causes intense luteinization of the ovaries and complete lobulo-alveolar development of the gland but no secretion. If the stimulated ovaries are excised, secretion will follow about thirty-six hours later and the gland will then present the appearance of full development.

It is well recognized that estrogen in physiological doses is only capable of stimulating extension and branching of the ducts of the mammary gland of the rat (Astwood and Geschickter, 1938). Therefore, one might deduce from these findings that progesterone or a combination of estrogen and progesterone is responsible for lobulo-alveolar development in the mammary gland of the rat. Removal of the luteinized ovaries followed by secretion within the alveoli is a noteworthy example of a withdrawal effect. It has been amply demonstrated that estrogen can suppress lactation (Edelmann and Gaunt, 1941) but, also, it has been shown that large doses of estrogen administered to an oophorectomized rat will cause formation of cysts of various sizes arising from the termini of the ducts and filled with secretion (Astwood and Geschickter, 1938). Therefore, the part that estrogen plays in the secretion of the mammary gland is still controversial. It would not be unreasonable to suppose that progesterone withdrawal is responsible for the initiation of lactation just prior

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to parturition in view of experimental evidence which shows that the level of progesterone drops at the end of gestation. Bachman and co-workers (1940) demonstrated a pre-labor drop in pregnanediol excretion, and Lyon (1946) likewise found that the pregnanediol excretion in human beings during the last two weeks of pregnancy declined and that the fall became abrupt during the last six days prior to the onset of labor. Other investigators (Atkinson and Hooker, 1945; Atkinson and Leathem, 1946) have examined the histological changes in the uterus of the mouse from day to day in pregnancy and lactation and they have found that, whereas a progesterone effect was predominant in the middle of pregnancy, the changes at the end of gestation were characteristic of estrogen stimulation.

In 1931, Turner and Schultze injected a corpus luteum extract into ovariectomized rats without any effect on the mammary gland, but combined administration of an estrogen from the urine of pregnant cows and the corpus-luteum extract in spayed female rats produced lobule formation. Astwood and Geschickter (1938) repeated this experiment later with crystalline progesterone alone and together with estrone; the largest dose, 1 mg. of progesterone and 5 μg. of estrone daily for eight days, induced no demonstrable lobulo-alveolar growth. In 1948, Meites and Turner reported that limited lobulo-alveolar development but no secretion could be obtained in oophorectomized rats after injection of 500 IU of estrone with 15 mg. progesterone every day for ten days. Selye (1940) claimed to have obtained extensive lobular development in the mammary gland of oophorectomized rats by injection of 15 mg. of progesterone alone every day for ten days. Hamolsky and Sparrow (1945) gave various combinations of estradiol, progesterone, and relaxin after pretreatment with estradiol and found that lobulo-alveolar development was more advanced at the end of nine days among spayed rats that received relaxin with estrogen and progesterone than in animals given only estrogen and progesterone. It is easily seen that there is a discrepancy among the findings pertaining to the relative importance of certain reproductive hormones in influencing the mammary gland.

This paper presents results of an investigation of the influence of estrogen and progesterone in bringing about complete lobulo-alveolar development of the mammary gland and mammary secretion in immature, ovariectomized rats.

MATERIAL AND METHODS

The animals used were rats of the Long Evans strain fed on Purina fox checkers. The mammary glands were studied in whole mounts; the partly fixed glands were dissected away from the subcutaneous tissue, fixed in Bouin's solution, stained in aqueous alum hematoxylin, cleared in xylol, and mounted in Canada balsam. Also, paraffin sections of many glands preserved in Bouin's solution and stained with hematoxylin and eosin were

TABLE 1. SUMMARY OF EXPERIMENT WITH CHORIONIC GONADOTROPIN

Daily dose of chorionic gonadotropin (I.U.)	Duration of treatment with chorionic gonadotropin (days)	Day killed
5	4	5
5	7	8
5	11	12
5	14	15
5	17*	19

* Ovariectomized on seventeenth day.

made. The whole mounts were most useful in viewing the gross architecture of the gland but, for actual detail, such as evidence of secretion, the hematoxylin and eosin sections proved to be more reliable. The endocrine preparations used were chorionic gonadotropin[2] from human pregnancy urine, estradiol benzoate[3] in sesame oil, aqueous suspensions of progesterone,[4] and progesterone[5] in sesame oil. No injections were given on Sundays and all injections were subcutaneous. The investigation was divided into four parts.

One group of immature, female rats was given chorionic gonadotropin according to the schedule outlined in Table 1. The animals were killed at intervals of several days after the start of injections so that the mammary glands could be removed for examination. It is to be noted that the ovaries

TABLE 2. SCHEDULE OF TREATMENT WITH ESTRADIOL AND PROGESTERONE

Daily dose of aqueous suspension of progesterone (mg.)	Duration of treatment with progesterone (days)	Dose of estradiol twice a week (mg.)	No. of estradiol injections	Day killed
—	—	0.1	6	22
—	—	0.001	6	22
4	21	—	—	22
15 (in oil)	12	—	—	13
4	21	0.1	6	22
4	21	0.001	6	22

were excised on the seventeenth day and the mammary glands were removed two days later. The second group of immature, female rats were oophorectomized and then given estradiol benzoate twice a week and progesterone daily, singly and together in various doses, for twenty-one days as indicated in Table 2. The third group of immature, female rats were also oophorectomized and then given a combination of estradiol benzoate twice a week and progesterone daily for seventeen days. No more progesterone was given after the seventeenth day and the last estradiol injection was administered on the eighteenth day. This experiment is summarized in Table 3. Rats were killed and glands were removed on each of five successive days after cessation of progesterone injections. The fourth group of animals was composed of nor-

[2] "Korotrin," Winthrop Chemical Company.
[3] "Progynon B," Schering Corporation.
[4] Parke, Davis, and Company.
[5] "Proluton." Schering Corporation.

TABLE 3. SCHEDULE OF TREATMENT WITH ESTRADIOL AND PROGESTERONE FOLLOWED BY PROGESTERONE WITHDRAWAL ON THE EIGHTEENTH DAY

Daily dose of progesterone in oil (mg.)	Duration of treatment with progesterone (days)	Dose of estradiol twice a week (mg.)	No. of estradiol injections	Day killed	Day of progesterone withdrawal on day killed
5	17	0.166	5	18	1
5	17	0.166	6	19	2
5	17	0.166	6	20	3
5	17	0.166	6	21	4
4 (in aqueous suspension)	17	0.100	6	21	4
5	17	0.166	6	22	5

mal female pregnant rats whose mammary glands were removed on the eleventh, seventeenth, eighteenth, nineteenth, twentieth, and twenty-first day of gestation and first day post-partum after suckling had begun.

RESULTS

Part I. Effect of Chorionic Gonadotropin. Daily injections of 5 IU chorionic gonadotropin into immature rats resulted in marked ovarian enlargement from the formation of numerous corpora lutea. The vagina in all cases opened by the fifth day of treatment and all smears were characteristic of estrus at this time. Subsequently, diestrus spreads for the most part were obtained. A gland on the fifth day after treatment simply showed extension of the duct tree and increased branching typical of an estrogen effect. On the eighth day after treatment the gland presented a different appearance entirely. At the termini of the ducts clusters of alveoli had formed. On the twelfth day after injection, larger clusters of alveoli were seen. The alveoli were packed closely together, thus giving the whole gland a denser appearance (Fig. 1). Lobule formation of these alveolar clumps was definite. The mammary gland on the fifteenth day after treatment showed similar changes. There was no gross evidence of secretion in any of these glands and no significant difference in size. A mammary gland on the nineteenth day two days after oophorectomy revealed doubling in size over the other glands and marked distention of alveoli with secretion (Fig. 2). It had not been possible to express milk from the gland prior to death.

Part II. Effect of Estradiol and Progesterone on Immature Oophorectomized Rats. 0.1 milligram of estradiol benzoate, given twice a week for three weeks, resulted in glands composed of cysts of all sizes containing secretion, irregular dilatation of the ducts, and stunted growth (Fig. 3). One-thousandth milligram of estradiol benzoate, administered in the same manner, induced extensive growth, arborization of ducts, and beginning cyst formation (Fig. 4). The administra-

tion of 4 mg. of progesterone daily for twenty-one days caused no detectable change in the mammary gland. A larger dose of 15 mg. daily for twelve days also appeared to have no effect (Fig. 5). It is interesting to contrast the appearance of the gland of a rat that was kept separately while receiving 4 mg. of progesterone daily for twenty-one days with one of a rat that, while being given the same dose of progesterone, was placed in a cage with animals being given estradiol. In the gland of the rat placed with other animals getting estradiol, incomplete lobulo-alveolar development occurred (Fig. 6). There was no change in the gland of the rat that was kept apart while getting progesterone. It is supposed that estradiol was obtained from the other animals that were receiving this substance. Combined treatment with estradiol benzoate twice a week, and 4 mg. of progesterone daily over a period of twenty-one days, resulted in complete lobulo-alveolar development but no secretion (Fig. 7). The gland of the rat that was given 0.1 mg. of estradiol benzoate with 4 mg. of progesterone proved to have more solidly packed alveoli than the gland of the rat that received only 0.001 mg. of the estrogen with 4 mg. of progesterone.

Part III. Effect of Progesterone Withdrawal Following Estradiol and Progesterone Administration in Immature Oophorectomized Rats

The withdrawal of progesterone on the eighteenth day after attaining complete lobulo-alveolar development with estradiol and progesterone was followed on the twentieth day, or the third day of withdrawal, by beginning secretion within the alveoli. Secretion reached a maximum on the twenty-first day, or the fourth day of withdrawal, and milk could be expressed through the nipple. Secretion began to recede on the twenty-second day. A better secretory response and more prominent nipples were obtained in those rats that received with the estradiol the aqueous suspension of progesterone rather than progesterone in oil. On the eighteenth day, or first day of withdrawal, clumps of closely packed alveoli were found (Fig. 8). Each alveolus was composed of a ring of high cuboidal epithelium with granular cytoplasm without secretion in the lumen. There was a moderate amount of fat tissue supporting the epithelial structures. Except for a slight increase of fat around the gland, the gland excised on the nineteenth day did not differ from the one on the eighteenth day. On the twentieth day, or third day of withdrawal, there was marked increase in the density of the gland, slight increase in size and more fat in the glandular region greatest around the base of the gland and least in the region of the nipple. Microscopically, there was beginning secretion within the alveoli, and there were numerous small fat globules within the cytoplasm of the epithelial cells, as well as in the material secreted into the lumen (Fig. 9). By the twenty-first day, there was even more secretion which was widespread throughout the gland, and milk could be expressed through the nipple. At the same time, the epithelial cells had flattened suggesting a massive discharge of material

which they had previously been storing. Fat globules were again seen in the cytoplasm and in the material secreted (Figs. 10 and 11). As will be seen later, this gland was similar in microscopic appearance to the mammary gland on the twenty-first day of pregnancy just before parturition. On the twenty-second day, the gland was less thick and secretion had begun to recede.

Part IV. Mammary Gland Changes During the Last Half of Pregnancy. By the eleventh day of gestation, alveoli in small clusters were beginning to form in the central portion of the gland while ducts only with their small end buds still occupied the periphery. Hematoxylin and eosin sections showed collapsed ducts, a few small groups of alveoli, and abundant fat tissue. Glands removed on the seventeenth day showed a greater profusion of alveolar clumps supported in fatty tissue. Hematoxylin and eosin sections showed no evidence of secretion. Excision of the gland on the eighteenth day revealed incomplete lobulo-alveolar development. There was a rare alveolus filled with secretion, but, for the most part, no secretory changes had taken place. Glands removed on the nineteenth and twentieth days showed pronounced lobulo-alveolar development, alveoli composed of cuboidal epithelium with markedly vesicular cytoplasm, and very slight secretion in some areas. By the twenty-first day, widespread secretion had taken place and there was marked reduction in height of the epithelium to a low cuboidal state (Fig. 12). Small fat globules were present in the cytoplasm as well as in the material secreted. Milk could not be expressed at this time. These changes were similar to those found in the glands of oophorectomized rats that had been given estradiol and progesterone followed by progesterone withdrawal after lobulo-alveolar development had been attained (Fig. 10). One day post-partum, after suckling had begun, there was marked distention of the alveoli with very little supporting fatty tissue seen. The alveolar epithelium was very flat and apparently broken in places so that a number of the lumina of the alveoli were connected.

DISCUSSION

There is no longer any uncertainty that the mammary gland is under hormonal control, but there is still much to be learned about the interaction of the specific hormones involved. It is felt that the experiments performed have shown that lobulo-alveolar development as extensive as that seen in normal pregnancy in the rat mammary gland can be produced by estrogen and progesterone. To date, this appears to be the greatest amount of lobulo-alveolar development obtained in the spayed rat with these hormones. The most advanced changes were obtained with 0.1 mg. of estradiol benzoate with 4 mg. of progesterone. This dose of estrogen may be excessive under normal circumstances but, during pregnancy, this amount might well lie within the physiological range.

That prolactin plays an important role in lactation appears to have been well established (Meites and Turner, 1948). However, the manner in which its action is coordinated with the ovarian hormones has been largely a matter of conjecture. It has been shown that estrogen increases the concentration of prolactin in the anterior pituitary (Meites and Turner, 1948). It is well established, too, that prolactin can stimulate secretion in the mammary gland, provided the latter is properly developed (Turner, 1948). The secretory action of prolactin is probably a direct one as demonstrated by Lyons (1942), who by injecting prolactin into single milk ducts obtained local sector lactation in the rabbit's mammary gland, which had been developed by estrogen and progesterone. In addition to this function, it has been shown that Prolactin causes luteal tissue to secrete progestin (Evans *et al.*, 1941), and in all probability, prolactin and the hypophyseal luteotrophin described by Astwood (1941) are identical. Stimulation of luteal tissue by prolactin to form progesterone, as well as production of mammary gland secretion by prolactin, are difficult to reconcile in view of the experimental evidence of inhibition of secretion by progesterone. However, these two effects of prolactin may be resolved if one examines the sequence of events in normal pregnancy. During the first half of pregnancy in the rat, corpus luteum function is maintained by the anterior pituitary, probably through the secretion of prolactin, but no mammary secretion occurs. This may either be a result of interference by progesterone or because the mammary gland has not reached the proper stage of development to be stimulated in this manner. During the last half of gestation in the rat, the placenta assumes the work of stimulating the function of the corpus luteum by secreting a chorionic luteotrophin which thus enables continued progesterone formation (Astwood and Greep, 1938). Apparently, near term, placental function and therefore progesterone formation decreases. In the human being, during the last two-thirds of pregnancy, the placenta secretes estrogen and progesterone. As a result of progesterone withdrawal prior to parturition, prolactin,

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FIG. 1. (top) Mammary gland of an immature female rat treated for twelve days with 5 IU chorionic gonadotropin. ×25.

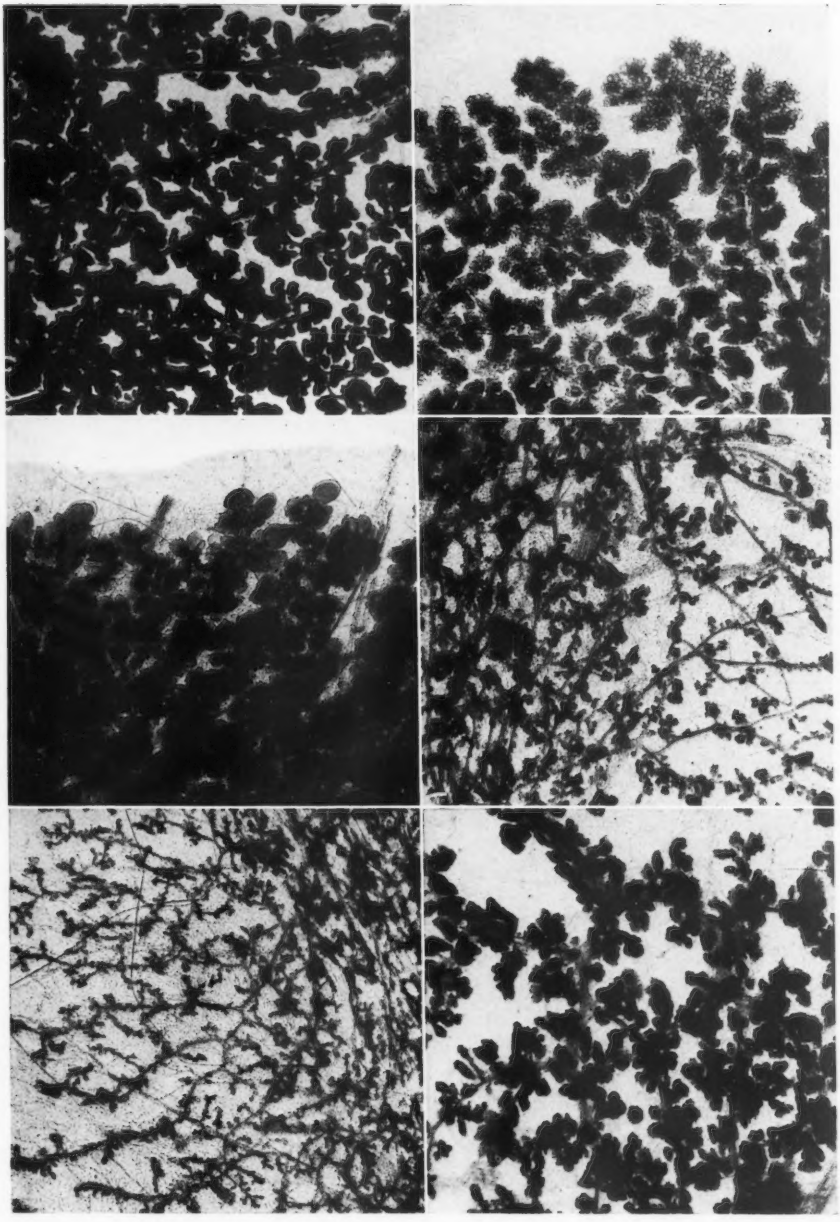
FIG. 2. (top right) Mammary gland of an immature female rat treated for seventeen days with 5 IU chorionic gonadotropin, oophorectomized on the seventeenth day and killed two days later. ×25.

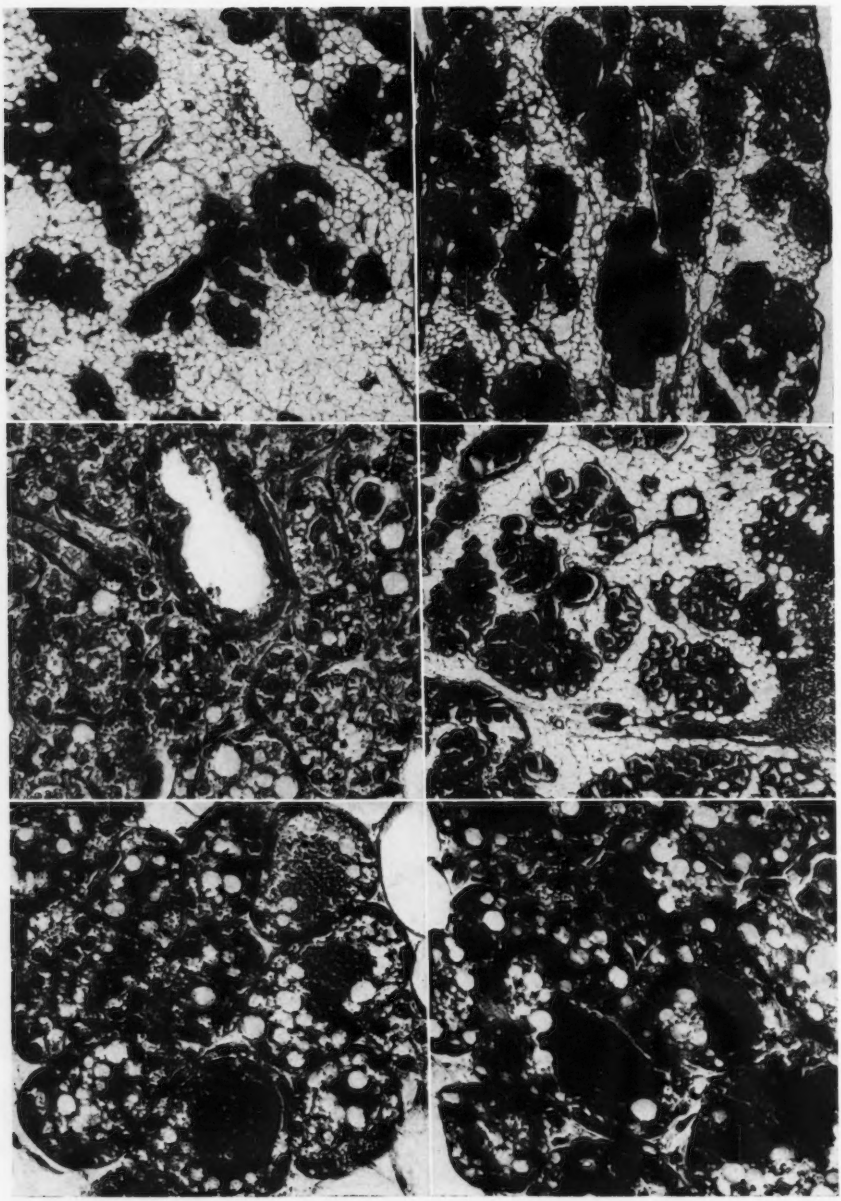
FIG. 3. (center left) Mammary gland of an immature spayed female rat treated twice a week for three weeks with 0.1 mg. estradiol benzoate. ×25.

FIG. 4. (center right) Mammary gland of an immature spayed female rat treated twice a week for three weeks with 0.001 mg. estradiol benzoate. ×25.

FIG. 5. (bottom left) Mammary gland of an immature spayed female rat treated daily for twelve days with 15 mg. progesterone in oil. Animal was kept in a separate cage while receiving progesterone. ×25.

FIG. 6. (bottom right) Mammary gland of an immature spayed female rat treated daily for twenty-one days with 4 mg. progesterone in aqueous suspension. While getting progesterone, rat was kept in a cage with other animals being given estradiol. ×25.





perhaps stimulated by estrogen, is then able to exert its secretory effect upon the mammary gland. It would appear, therefore, that secretion in the mammary gland is dependent upon the proper balance between prolactin and progesterone. By raising the prolactin to progesterone ratio, either by increasing prolactin or by decreasing progesterone, one, therefore, might induce secretion. In pregnancy, suppression of secretion is probably due to the high concentration of circulating progesterone. As a result of its abrupt fall at the end of gestation, prolactin is then able to evoke a secretory response in the gland.

It has been ascertained that estrogen requires the anterior hypophysis in bringing about growth and secretion (Leonard and Reece, 1942). The type of growth that estrogen induces in intact animals varies with the species. In the cow, goat, and guinea-pig, complete lobulo-alveolar development follows estrogen administration alone, while in the rat, mouse, and rabbit, only duct growth occurs, progesterone being necessary in addition for lobulo-alveolar growth. The nature of the pituitary factor essential for mammary growth is not known, but in all probability it is different from prolactin (Greep and Stavely, 1941).

Progesterone is essential for the maintenance of pregnancy in all mammals but it is not needed, as mentioned above, for lobulo-alveolar development of the mammary gland in all species. However, the presence of progesterone in high concentrations during pregnancy seems to be the prime factor in inhibiting lactation in mammals. The mechanism of this inhibition is not known, but it is entirely possible that it acts locally on the epithelial cells. In the rat, progesterone not only may prevent discharge of cellular material, but it also acts in conjunction with estrogen in promoting lobulo-alveolar growth. In view of the fact that prolactin also seems to act locally, it might act to release materials from the cytoplasm previously held in check by progesterone.

FIG. 7. (top) Mammary gland of an immature spayed female rat treated with 0.1 mg. estradiol twice a week and 4 mg. progesterone in aqueous suspension over a period of twenty-one days. Unfortunately, the photograph taken of the section was not representative of the entire field which included a greater number of clusters of alveoli. ×100.

FIG. 8. (top right) Mammary gland of an immature spayed female rat on first day of withdrawal of progesterone after attaining lobulo-alveolar development with estrogen and progesterone. ×100.

FIG. 9 (center left) Mammary gland of an immature spayed female rat on third day of progesterone withdrawal after attaining lobulo-alveolar development with estrogen and progesterone. ×800.

FIG. 10 (center right) Mammary gland of an immature spayed female rat on fourth day of progesterone withdrawal after attaining lobulo-alveolar development with estrogen and progesterone. ×100.

FIG. 11. (bottom left) Same as Figure 10. ×800.

FIG. 12. (bottom right) Mammary gland of a normal pregnant rat on the twenty-first day of gestation. ×800.

Experiments on hormonal inhibition of established lactation have yielded variable results. Hypophysectomy in the rat during the second half of gestation does not prevent the onset of lactation just after parturition (Selye *et al.*, 1933). However, this secretion lasts only a few hours. No satisfactory explanation of this phenomenon has been presented. Hypophysectomy stops the flow of milk promptly when the operation is performed at any time after delivery (Selye *et al.*, 1933). It has been reported that estrogen can inhibit lactation in oophorectomized rats, but it is more effective in intact rats (Edelmann and Gaunt, 1941). Gonadotropins, likewise, (Edelmann and Gaunt, 1941) mediate their lactation-inhibiting properties through the ovary, which suggests that both hormones function by virtue of a direct or indirect luteotrophic action. Thus far, progesterone administration has usually failed to inhibit milk flow (Edelmann and Gaunt, 1941). The flow of milk post-partum arises as a result of local stimulation of the nipple by suckling, and this may in part be mediated by an increased elaboration of prolactin. In view of the probable individual variation in response to suckling, one should not be surprised to obtain variable degrees of inhibition by hormones. The response of lactating human beings to estrogen is most inconsistent (Birnberg *et al.*, 1947). Sometimes no effect has been observed. In others only a relief of congestion has been experienced, and, in a few, a decrease of flow has been obtained. In the last analysis it would appear that the predominant mammary effect during gestation until just prior to parturition is that of progesterone which, while furthering lobulo-alveolar development in most species, interferes with secretory action of prolactin. The hormonal effect predominating during the suckling period is prolactin, which may be able to override any effect by progesterone or substances such as estrogen or gonadotrophin that simulate the secretion of progesterone.

SUMMARY

Secretion in the mammary gland of the normal pregnant rat appears about two days prior to parturition. Chorionic gonadotropin causes intense luteinization of the ovaries and complete lobulo-alveolar development of the mammary gland in the intact rat but no secretion. If the luteinized ovaries of rats treated with chorionic gonadotropin are excised, secretion in the developed mammary gland will follow in forty-eight hours. Estrogen and progesterone when given together to ovariectomized immature rats in suitable doses cause complete lobulo-alveolar development of the mammary gland but no secretion. Withdrawal of progesterone after obtaining lobulo-alveolar development with estrogen and progesterone in ovariectomized immature rats results in mammary gland secretion which is greatest on the fourth day of withdrawal. The occurrence of secretion appears to

be dependent upon the ratio of prolactin to progesterone acting upon the mammary gland.

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THE PARADOXICAL EFFECTS OF THIOCYANATE AND OF THYROTROPIN ON THE ORGANIC BINDING OF IODINE BY THE THYROID IN THE PRESENCE OF LARGE AMOUNTS OF IODIDE

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IT HAS been demonstrated by Wolff and Chaikoff (1948) that the administration of a large dose of iodide to the intact rat inhibits the organic binding of iodine by the thyroid gland. In the experiments reported, the inhibition was correlated with the plasma iodide concentration, and the effective values were found to be above 20–35 μg. per 100 cc.

The ability of thiocyanate and of thyrotropin to affect the iodide concentrating power of the thyroid offered a means for manipulating the iodide content of the gland without changing the plasma iodide concentration appreciably. Thiocyanate markedly inhibits the capacity of the thyroid to concentrate iodide ion (Franklin, Chaikoff, and Lerner, 1944; VanderLaan and VanderLaan, 1947), and, at physiological plasma iodide concentrations, slows the process of synthesis of organic iodine compounds (Wolff, Chaikoff, Taurog, and Rubin, 1946). Thyrotropin increases the iodide concentrating power (Stanley and Astwood, 1949) and has the effect, at ordinary iodide values, of increasing the rate of organic binding (Leblond and Sue, 1941; Morton, Perlman, and Chaikoff, 1941). Paradoxical effects on the rate of organic synthesis would be expected from the administration of thiocyanate and of thyrotropin in the presence of high plasma iodide concentrations if the iodide inhibition were a function of the iodide concentration in the thyroid gland.

METHODS

Long-Evans rats on a diet of Purina Fox Checkers were used. When pretreated with propylthiouracil, the drug was given in a 0.03 per cent concentration in Purina Mink Chow. The low-iodine diet used in some experiments was made from the following formula: wheat gluten, 360 Gms.; yellow corn meal, 1400 Gms.; dried brewer's yeast, 100 Gms.; salt, 20 Gms.

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All determinations were made by radioactivity measurements. Iodide, as potassium iodide, was labelled with radioactive iodide (I^{131}) and the specific activity of this solution (cts./sec./μg.) determined. The iodide solution was injected intraperitoneally. The amount of iodide injected was always many times greater than the total iodide content of the animal. The latter was therefore neglected in all calculations. Potassium thiocyanate, when used, was given at the same time and by the same route as the iodide. Thyrotropin was injected subcutaneously.

At the termination of the experimental period, the rat was anesthetized with ether, the chest opened, blood obtained for plasma I^{131} content by heart puncture, and the animal killed by exsanguination. The thyroid glands were then removed immediately, weighed, and transferred to glass grinders where they were homogenized in 0.5 cc. saline by grinding between fitted ground glass surfaces. The homogenate was quickly transferred to a centrifuge tube containing 0.5 cc. of 40 per cent trichloracetic acid and a drop of 0.1N sodium thiosulphate solution. The grinder was rinsed twice with 0.5 cc. saline and the washings added to the centrifuge tube. The protein precipitate containing the organically bound I^{131} was separated from the supernatant containing the inorganic I^{131} by centrifugation. The protein was washed with 1.0 cc. of 5 per cent trichloracetic acid, centrifuged again, and the liquid added to the first supernatant fraction. Plasma and the supernatant liquid were counted directly; the thyroid protein was dissolved in a strong potassium hydroxide solution and then counted.

In thyrotropin experiments 1, 2, and 3 of Table 2, only the iodine bound during the experiment was determined. Iodide was discharged from the thyroid by injecting 10 mg. potassium thiocyanate one hour prior to killing the animal and the entire gland counted.

The radioactivity measurements were made in the following manner. 1.0-cc. liquid samples were counted in standardized 10-cc., flat-bottomed vials. The base of the vial was placed in contact with a shielded and firmly fixed Geiger-Mueller tube through a hole in the lead shield cut exactly to size for the standard vial. The Geiger-Mueller tube was a brass-encased, gamma-ray counting tube of high efficiency (Sylvania, Type GG306). In this constant geometrical arrangement, one microcurie of I^{131} in 1.00 cc. produced 272 counts/second.

EXPERIMENTAL AND RESULTS

It was noted early in the course of the experiments that considerably more iodide was required to inhibit organic binding in the stock rats in our colony than had been required for the animals used by Wolff and Chaikoff. In experiment 1, Table 1, satisfactory inhibition was produced by 600 μg. of iodide during a six and one-half hour experiment, and thiocyanate acted to remove the iodide block and increase the rate of synthesis of organic iodine compounds. In the subsequent experiments shown in Table 1, rats were used a number of days after a course of treatment with propylthiouracil, and while being maintained on a diet relatively low in iodine. Propylthiouracil was used to produce hyperplastic thyroid glands, and the low-iodine diet to maintain the hyperplastic state after the propylthiouracil was

TABLE 1. EFFECT OF THIOCYANATE ON THE ORGANIC BINDING OF IODINE IN RATS INJECTED WITH LARGE DOSES OF IODIDE

Expt. No.	Iodide injected μg.	KSCN injected Mg.	Experimental period hours	Rat wt. gm.	Thyroid wt. mg.	Iodide conc. ratio thyr:se	Thyroid iodide mg./100 gm. thyroid tissue	Iodine organically bound during experiment μg.
I	600	0		87	8.25	—	1.63	.152
	600	0		89	6.5	—	1.39	.06
	600	0		90	10.1	—	1.89	.0567
	600	7.5	6.5	89	8.15	—	0.291	.176
	600	7.5		86	8.0	—	0.348	.177
	600	7.5		87	9.2	—	0.522	.243
	600	7.5		87	9.1	—	0.345	.551
II	100	0		120	43.3	204.1	9.9	.097
	100	0		105	25.4	228.5	10.2	.056
	100	1.0	4.0	125	41.8	53.4	3.5	.15
	100	1.0		98	42.9	32.0	2.8	.13
	100	30.0		115	44.9	3.1	.45	.22
	100	30.0		145	33.3	3.0	.24	.14
Group II: Propylthiouracil 13 days, with low-iodine diet during last 36 hours. Continued on low-iodine diet and used 3 days after propylthiouracil stopped.								
III	100	0		155	36.0	205.5	7.3	.17
	100	0		140	33.2	159.8	8.0	.099
	100	2.0	4.0	105	39.9	30.5	3.3	.069
	100	2.0		145	32.4	29.2	2.3	.22
	100	30.0		180	39.9	3.2	0.16	.35
Group III: Propylthiouracil 17 days, with low-iodine diet during last 2 days. Continued on low-iodine diet and used 3 days after propylthiouracil stopped.								
IV	150	0		170	41.7	86.2	4.9	.08
	150	0		173	35.1	128.0	6.0	.155
	150	7.5	5.0	177	36.9	8.2	0.69	.419
	150	7.5		175	27.8	16.0	1.01	.33
	150	30.0		185	39.7	3.3	0.2	.19
	150	30.0		165	28.3	2.5	0.22	.17
Group IV: Propylthiouracil 23 days, with low-iodine diet during last 2 days. Continued on low-iodine diet and used 4 days after propylthiouracil stopped.								
V	200	0		90	13.2	41.8	6.7	.051
	200	0		80	11.4	10.3	1.6	.061
	200	0		85	13.5	62.8	6.5	.064
	200	7.5		75	9.9	5.3	1.1	.083
	200	7.5	4.5	82	12.2	3.3	0.46	.32
	200	7.5		92	19.4	6.0	0.44	.23
	200	30.0		75	10.7	2.4	0.22	.066
	200	30.0		75	10.4	1.7	0.2	.061
	200	30.0		85	12.8	2.8	0.23	.102
Group V: Propylthiouracil 10 days, with low-iodine diet during last 4 days. Continued on low-iodine diet and used 12 days after propylthiouracil stopped.								

stopped. The hyperplastic thyroid glands thus produced were readily inhibited by smaller doses of iodide than were required by non-hyperplastic glands. These enlarged glands were more like those studied by Wolff and Chaikoff, which were probably hyperplastic as a result of a low-iodine diet. The pretreated animals maintained thyroid:serum iodide ratios which were much higher than those of the untreated animals, and were in the range of the ratios reported by the previous workers.

Table 1 and Figure 1 demonstrate that, following a large dose of iodide, an inverse correlation existed between the iodide concentration in the thyroid and the quantity of iodine organically bound. Under the conditions of the experiment, organic binding was increased by thiocyanate.

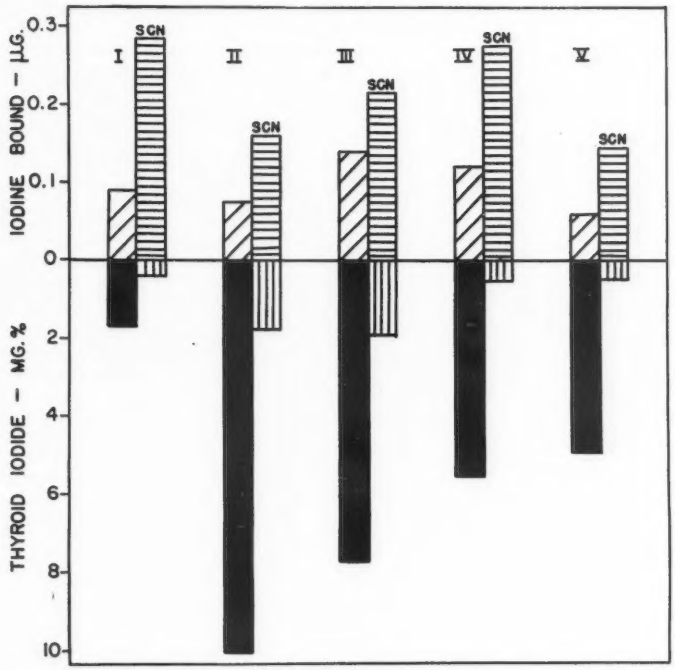

FIG. 1. Thiocyanate effect. Illustration of data from Table 1. The columns on the left for each experiment represent the animals which received no thiocyanate, and those on the right, the thiocyanate-treated animals. The thyroid iodide concentrations and the values for iodine organically bound have been averaged in each experiment for the thiocyanate group and the untreated group. The average bound iodine is represented by the columns above the line, and the average thyroid iodide concentrations by the columns below the line. It may be seen that a large thyroid iodide concentration is associated with a small amount of iodine bound, and that when the thyroid iodide is decreased with thiocyanate, iodine binding is increased.

In converse experiments, rats were injected with amounts of iodide which, though large, were not large enough to block iodine binding. A block was effected under these conditions, however, by pretreatment with thyrotropin. The data are shown in Table 2 and illustrated in Figure 2.

DISCUSSION

The experimental results indicate that high concentrations of iodide have a marked inhibiting effect on iodine binding by the thyroid, as reported by Wolff and Chaikoff in the rat, and as subsequently observed in man (Stanley, 1949). It further appears that the inhibition is a function of the iodide ion concentration in the thyroid gland. Smaller doses of iodide were required to produce inhibition in animals maintaining a higher thyroid:serum iodide ratio

TABLE 2. EFFECT OF THYROTROPIN ON THE ORGANIC BINDING OF IODINE IN RATS INJECTED WITH LARGE DOSES OF IODINE

Expt. No.	Iodide injected μg.	Thyrotropin injected Mg.	Experimental period hours	Rat wt. gm.	Thyroid wt. mg.	Iodide conc. ratio thyr:se.	Thyroid Iodide mg./100 gm. thyroid tissue	Iodine organically bound during experiment μg.
I	42	0	6.5	33	—	—	—	.60
	42	0		33	—	—	—	.43
	42	15		32	—	—	—	.047
	42	15		35	—	—	—	.054
Group I: Thyrotropin injected 10 hours before iodide.								
II	45	0	6.5	50	—	—	—	.78
	45	0		65	—	—	—	.71
	45	15		70	—	—	—	.64
	45	15		50	—	—	—	.1
Group II: Thyrotropin injected 10 hours before iodide.								
III	35	0	6.25	38	—	—	—	.57
	35	0		32	—	—	—	.38
	35	0		33	—	—	—	.29
	35	15		32	—	—	—	.08
	35	15		38	—	—	—	.31
	35	15		30	—	—	—	.12
Group III: Thyrotropin injected 9½ hours before iodide.								
IV	42	0	6.5	31	4.5	—	0.22	.28
	42	0		33	4.5	—	0.51	.32
	42	0		32	5.0	—	0.43	.49
	42	15		30	5.0	—	0.54	.04
	42	15		32	4.9	—	0.88	.17
Group IV: Thyrotropin injected 10 hours before iodide.								
V	60	0	4.0	140	10.8	24.1	0.69	.553
	60	0		140	9.6	33.1	1.06	.52
	60	0		100	6.3	11.9	0.51	.22
	60	20		130	11.7	54.8	1.54	.108
	60	20		130	11.4	44.0	1.46	.051
	60	20		120	9.8	45.6	1.6	.083
Group V: 10 Mg. Thyrotropin injected 84 hours and 10 mg. 24 hours before iodide.								

than in those with a lower ratio. Suppression of the iodide concentrating mechanism by thiocyanate released the gland from the inhibiting effect, whereas increasing the iodide concentrating power of the gland with thyrotropin accentuated the iodide inhibiting action.

In the thiocyanate experiments, plasma iodide concentrations at the end of the experimental period were almost invariably above 35 μg. per 100 cc., the concentration that inhibited binding in all the animals studied by Wolff and Chaikoff. There was essentially no difference in the plasma iodide values for the thiocyanate treated and non-treated groups. The increase in iodine binding in the thiocyanate treated animals, which can be correlated with the thyroid iodide concentration, cannot be correlated with the plasma iodide concentration.

It becomes of interest to inquire into the relationship between the iodide concentration in the thyroid and the quantity bound when the iodide values are below the inhibitory level. If results such as those shown in Figure 3 prove to be the usual relationship, it would appear that the rate of organic synthesis of iodine compounds increases with

increasing iodide concentrations. This would imply that a maximum value for iodine binding would be attained as thyroid iodide is increased, but that the further addition of iodide would then depress binding. An analysis of the data on the rat published by Wolff and Chaikoff (1948b) reveals that these are consistent with this concept, as are the data of Stanley on man.

The only action of thiocyanate on the thyroid gland that has been clearly demonstrated is a marked inhibition of the iodide ion concentrating power of the gland. When, as in Figure 4, organic synthesis

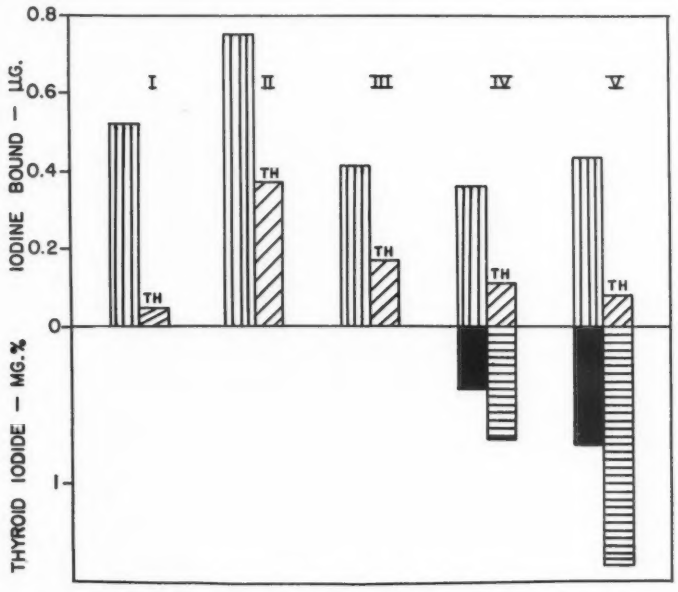

FIG. 2. Thyrotropin effect. Illustration of data from Table 2. Values have been averaged as for Figure 1, and columns arranged similarly. Thyroid iodide values were not determined in experiments I, II, and III.

is depressed by thiocyanate in the presence of low or moderately elevated plasma iodide values, it seems reasonable to relate the depression to the unavailability of iodide to the thyroid through the loss of its concentrating capacity.

It appears that even large amounts of thiocyanate cannot reduce the thyroid:serum iodide ratio below 1, and indeed, the ratio tends to remain somewhat above 1. When three rats weighing 100 ± 2 Gms. were each injected with 30 mg. of potassium thiocyanate, the thyroid:serum ratios one and one-half hours after the injection were found to be 1.4:1, 1.8:1, and 1.7:1. A high plasma level of iodide should therefore overcome the thiocyanate inhibiting effect on iodine binding if

this substance acted only on the iodide concentrating mechanism. The prevention of thiocyanate goiter with iodide (Astwood, 1943) suggests that such is the case. Experimental results at times, however, suggested the possiblity that large doses of thiocyanate could in addition affect organic synthesis directly. Such suggestion appears both in some of the data in Table 1, and in the data shown in Figure 5.

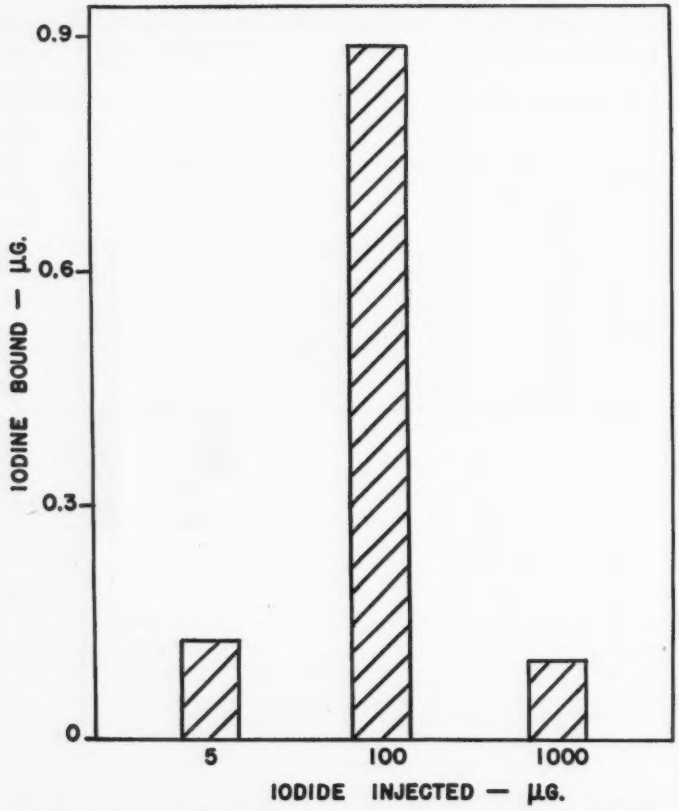

FIG. 3. The influence of iodide dosage on the amount of iodine bound. Rats weighing 82 ± 5 Gms. were injected with 5.0, 100, or 1000 μg. of iodide labelled with I^{131}. After 6¼ hours, the animals were injected with potassium thiocyanate to discharge thyroid iodide, killed 45 minutes later, and the radioactivity in the glands measured. The column for the 1000 μg. animals is probably about 33 per cent too high due to iodide remaining in the thyroid, as estimated from the results of iodine fractionations in other animals under the same conditions. Each column represents the average of two animals.

Figure 5 illustrates the effect of various amounts of thiocyanate on iodine binding in the presence of large amounts of iodide. When 100 μg. of iodide was used, an amount which was insufficient to block binding, a progressive decrease in binding occurred with increasing

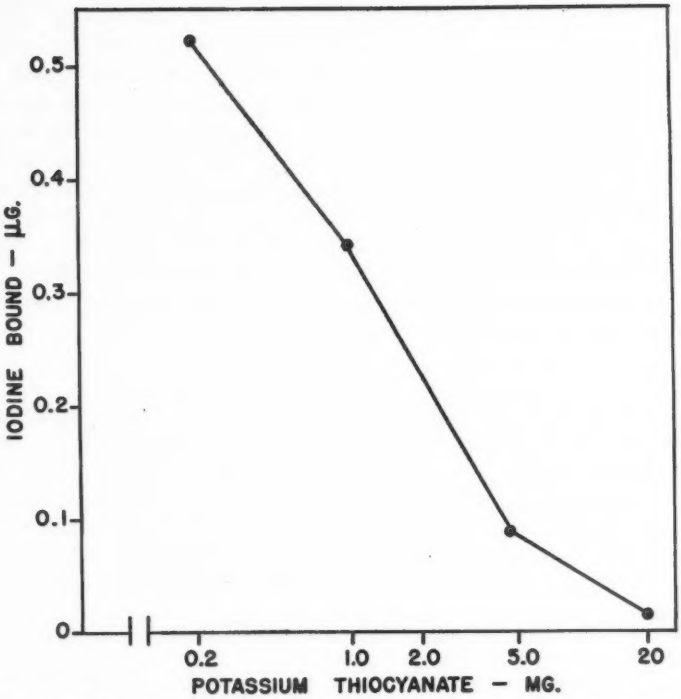

FIG. 4. Relationship of thiocyanate dosage to the quantity of iodine bound. Rats weighing 86 ± 6 Gms. were each injected with 15 μg. of labelled iodide and with the doses of potassium thiocyanate indicated on the abscissa. The same procedure was followed as in Figure 3. Each point represents the average of two animals.

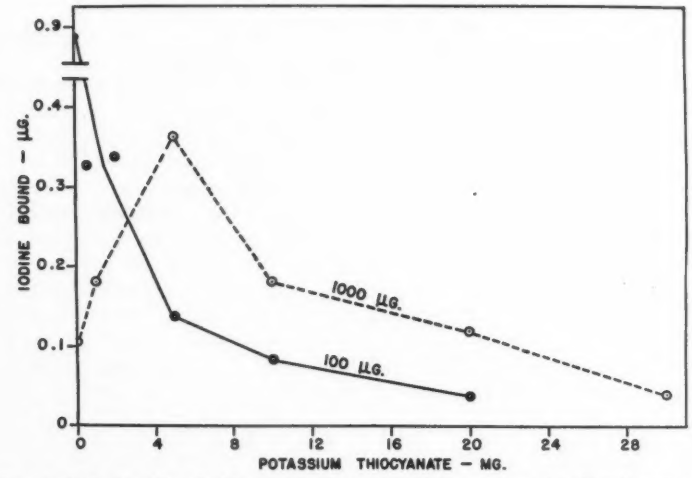

FIG. 5. Relationship of thiocyanate dosage to the quantity of iodine bound in the presence of large amounts of iodide. The rats weighed 85 ± 12 Gms. and the same procedure was followed as for Figure 3. Each point is the average of two animals.

amounts of thiocyanate. The results obtained with 1000 μg. of iodide indicate that this dose blocked binding and that the addition of thiocyanate acted to release the block, as in the experiments described earlier. However, with the larger doses of thiocyanate (20 and 30 mg.), binding was again depressed. Extremely low values were obtained for bound iodine in the group receiving 100 μg. of iodide and 20 mg. of potassium thiocyanate, and in the group treated with 1000 μg. of iodide and 30 mg. of potassium thiocyanate. It is difficult to account for such low values on the basis of thyroid iodide concentrations since these would be adequate for more rapid binding even if one assumed a thyroid: serum ratio as low as 1.

SUMMARY

The inhibition of iodine binding by the thyroid which occurs in the rat following a large dose of iodide is shown to be directly related to the iodide concentration in the thyroid gland. The usual effects of thiocyanate and of thyrotropin on the rate of organic binding of iodine by the thyroid can be reversed by the addition of large amounts of iodide. It is believed that the influence of these substances on the iodide concentrating power of the gland explains the paradoxical effects.

ACKNOWLEDGMENTS

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STUDIES ON THE FAT-MOBILIZING FACTOR OF THE ANTERIOR PITUITARY GLAND[1]

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ON THE bases of chemical and physiological evidence there has been general acceptance of six well-defined anterior pituitary hormones: growth hormone, thyrotrophin, corticotrophin, luteotrophin (lactogenic hormone), follicle-stimulating hormone, and luteinizing hormone. Anterior pituitary preparations have, however, been noted to cause a number of metabolic effects variously described as diabetogenic, glycotrophic, glycostatic, fat mobilizing, respiratory quotient lowering, ketogenic, pancreatotrophic, etc. It would be desirable to define more closely the hormones responsible for these various effects so as better to characterize the secretions of this complex endocrine gland.

This study deals with the hypophyseal factor which leads to an increase in liver fat; it was undertaken with the aim of determining whether this principle could be identified with any one of the six well-characterized hormones, or whether evidence could be adduced indicating a separate factor.

METHODS

The fat-mobilizing activity of a number of pituitary preparations was studied by the method of Campbell (1938). Immature female mice of the Swiss strain weighing 18 to 22 grams, fed on Purina mink chow, were given a single subcutaneous injection of the test substance and then fasted until they were killed with chloroform seven hours later. After injection the animals were placed in cages the floors of which were covered with wood shavings and permitted access to water. The room temperature averaged 25°C.

Immediately after death a 500-mg. portion of the liver was ground with approximately 4 gm. of anhydrous sodium sulfate in a dry mortar as described by Weil and Stetten (1947). The powdered preparation was then put into a 50-cc., clean, dry, screw-cap vial, the cap of which was lined with tinfoil and seated in cork. Thirty cubic centimeters of chloroform was added and the screw cap sealed. The preparation was then either turned end over end by a mechanical device at 50 to 60 revolutions per minute for sixteen hours or allowed to stand for seventy-two hours with intermittent shaking by hand.

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Ten cubic centimeters of the supernatant fluid was then filtered into a tared beaker, evaporated to dryness in a hot-air oven and weighed.

This method showed good correlation with the Soxhlet intermittent type of fat extraction on duplicate samples of mouse and rat livers of various fat concentrations. It had the advantage of simplicity of equipment and ease of operation.

Every effort was made to keep the preparation free from water as the presence of water prevented efficient extraction. Excess water could be determined by placing a pinch of anhydrous copper sulfate into the solution. A change of color to blue indicated water contamination.

Adrenalectomy was performed under ether anesthesia through a lumbar approach. All animals were discarded if the capsule of the adrenal was broken during removal. Adrenalectomized animals were given 0.9 per cent NaCl as drinking water and were used one week postoperatively. Thyroidectomy was done with ether anesthesia under a dissecting microscope. Propylthiouracil was given in a concentration of .03 per cent admixed with the food for two weeks prior to assay; this led to marked thyroid enlargement indicating an appreciable if not complete inhibition of thyroid hormone formation.

The soluble anterior pituitary extracts were prepared for injection by solution in physiological saline; the crude powders were suspended in saline with a homogenizer. All injections were given subcutaneously. When two separate substances were administered, they were given in different sites.

Each anterior pituitary preparation was tested over a wide dosage range; the resulting liver fat concentration was then plotted against the log of the dose. With most preparations a roughly linear dose-response relationship was noted over a limited range, and the potencies of different preparations were compared by reference to this portion of the curves.

RESULTS

About 500 separate determinations of liver fat concentration were done by the method described. The liver fat concentration of sixty normal control fasted mice ranged from 4 to 6 per cent and averaged 4.96 per cent. No difference was noted in the control animals whether untreated or injected with 1 cc. of 0.9 per cent NaCl solution.

All anterior pituitary preparations tested showed some activity, though the potencies varied between wide limits. The quantities of fat-mobilizing factor in the various preparations tested are shown in Figures 1 to 4.

Figure 1 represents the activity of acetone-dried pituitary powders of various species. These were similar in activity with the possible exception of pork pituitary, which was somewhat more active. Figures 2, 3, and 4 illustrate the fat-mobilizing effect of preparations of the pituitary which had been further purified. These refined preparations have been catalogued according to their activity into high, medium, and low potency groups.

A refined pituitary substance, Antuitrin T-607, was the most potent of the preparations tested. It was noted with this preparation

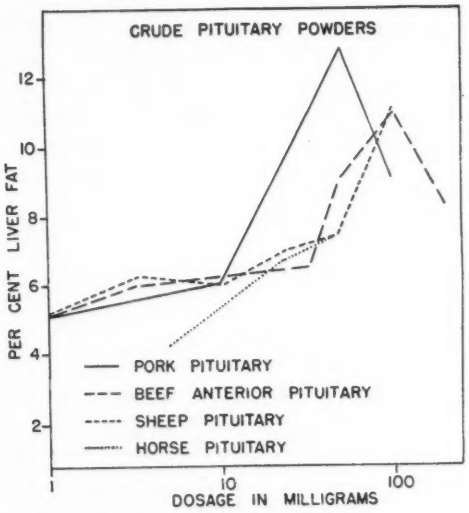

FIG. 1. Fat-mobilizing activity of crude, defatted pituitary powders. All preparations except beef anterior pituitary were derived from the whole pituitary.

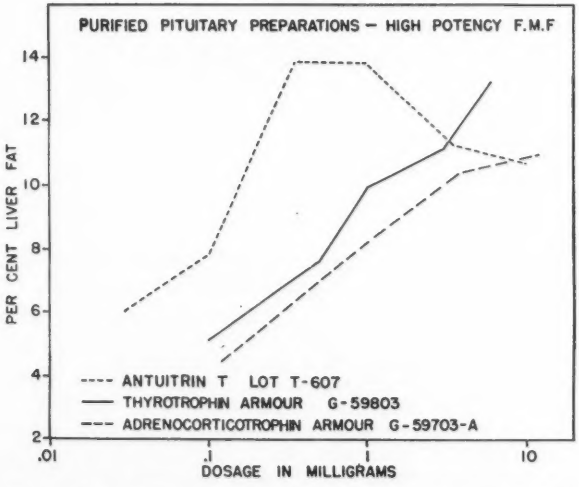

FIG. 2. Antuitrin T-607 contained the highest content of fat-mobilizing factor of the preparations tested. Thyrotrophin G-59803 possessed a high content of thyrotrophin and adrenocorticotrophin with a low assay of other factors. Adrenocorticotrophin G-59703-A contained only slightly higher adrenocorticotrophic activity than the above thyrotrophin. The latter two preparations had been subjected to sterilization filtration but still contained slightly greater potency of fat-mobilizing factor than the preparations shown in Figure 3.

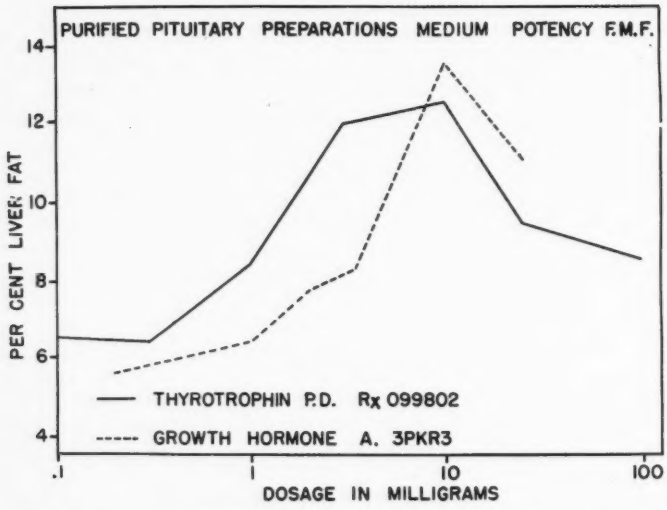

FIG. 3. Thyrotrophin (Parke-Davis R‡ 099802) possessed the highest thyrotrophic activity of any preparation tested.
Growth Hormone 3PKR3. Purified growth hormone (Armour).

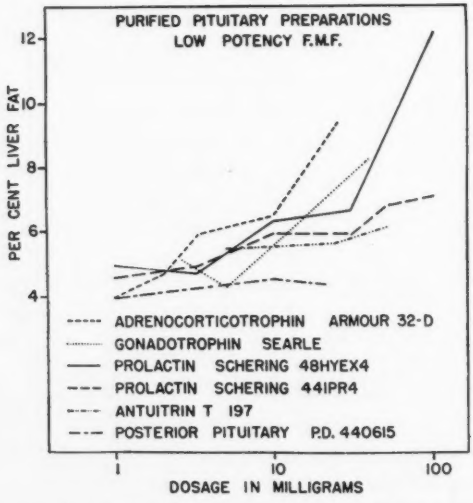

FIG. 4. Adrenocorticotrophin 32-D contained the highest adrenocorticotrophic activity of any preparation tested . . . (78 per cent of Armour Standard) with a moderately high assay of Prolactin (2½ I.U./Mgm.). Schering's Prolactin 48HYEX4 contained 20 I.U./Mgm. and Prolactin 441PR4 contained 7.5 I.U./Mgm.

that about 30 per cent of the activity was lost in five days when solutions of the material were kept at room temperature. B. subtilis was cultured from these solutions and may have accounted for the change of activity. The tests on the other preparations were made on freshly prepared solutions.

Saline suspensions of posterior pituitary showed no activity in the dosages used. Pitressin, 1 cc., (Parke-Davis), was quite toxic to the

TABLE 1. ADRENAL EFFECT ON FAT-MOBILIZING FACTOR

Number of animals		Per cent liver fat
Intact		
3	Lipo-Adrenal Cortex, 1 cc.	5.53
1	Lipo-Adrenal Cortex, .3 cc.	6.48
1	Lipo-Adrenal Cortex, .1 cc.	4.88
2	Eschatin, 1 cc.	6.09
2	Sesame Oil, .25 cc.	4.68
Adrenalectomized—7 Days Post-Operative		
3	Uninjected	4.36
2	Antuitrin T*, 2.5 mg.	5.61
2	Antuitrin T*, .5 mg.	6.15
1	Thyrotrophin (Parke-Davis), 20 mg.	3.00
2	Lipo-Adrenal Cortex, .5 cc.	3.48
2	Desoxycorticosterone Acetate in Sesame Oil, 2.5 mg.	5.2
3	Lipo-Adrenal Cortex, .5 cc. +Antuitrin T*, 2 mg.	9.42
2	Lipo-Adrenal Cortex, .25 cc. +Antuitrin T*, 2 mg.	8.73
4	Lipo-Adrenal Cortex, .05 cc. +Antuitrin T*, 2 mg.	7.18
2	Lipo-Adrenal Cortex, .01 cc. +Antuitrin T*, 2 mg.	8.37
2	Eschatin, 1 cc. +Antuitrin T*, 2 mg.	8.79
2	Eschatin, .5 cc. +Antuitrin T*, 2 mg.	5.94
8	Desoxycorticosterone Acetate in Sesame Oil, 2.5 mg. +Antuitrin T*, 2 mg.	7.83
2	Sesame Oil, .25 cc. +Thyrotrophin (Parke-Davis), 20 mg.	5.12

* Antuitrin T, Lot T-607 (Parke-Davis & Company).

mice and caused a slight rise in liver fat (6.24–6.6 per cent liver fat). These values are not considered significant because of the possible effect on the liver of the preservative, chloretone.

The maximum response was higher with some preparations than with others, suggesting that there might have been other factors modifying the fat-mobilizing effect. When the dosage was increased above that which gave a maximal increase in liver fat, the response progressively declined. This phenomenon is unexplained.

Under the conditions of this experiment adrenalectomy inhibited completely the response to the fat-mobilizing factor (Table I). The response of the adrenalectomized animals to this factor was restored by adrenal cortical extract. Lipo-Adrenal Cortex (Upjohn) was in excess of fifty times as effective as the aqueous adrenal extract used (Eschatin—Parke-Davis) in this particular function. The mobiliza-

tion of the oil in the preparation was found not to be the responsible agent as the injection of sesame oil alone was without effect. However, without added fat-mobilizing factor the adrenal cortical hormones did not produce fatty liver in either the intact or the adrenalectomized animal. The injection of Lipo-Adrenal Cortex with fat-mobilizing factor in the intact animal produced no substantial change in the shape of the response curve, except perhaps for a slight depression of the effect in the higher dosage range (Fig. 5). When desoxycorticosterone

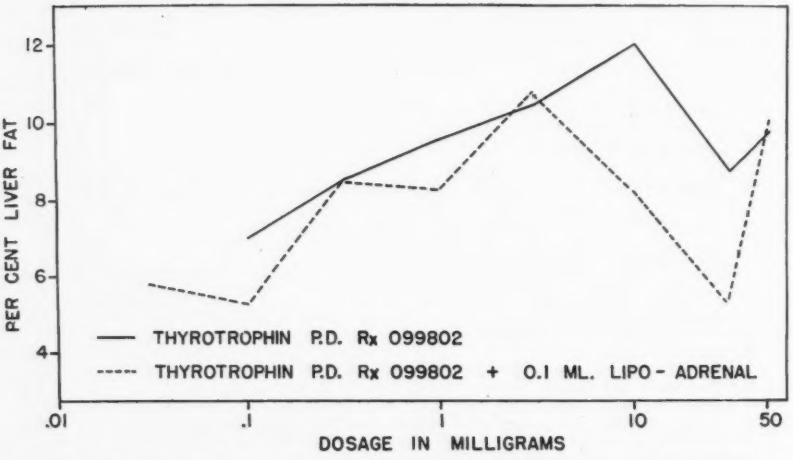

FIG. 5. The effect of adrenal cortical substance on fat-mobilizing factor activity in the intact fasted mouse. There was generally a slight depression of effect noted particularly in the higher dosage range.

acetate was used as replacement therapy in the adrenalectomized animals, a variable response to fat-mobilizing factor was obtained. Desoxycorticosterone acetate produced no change in the liver fat of adrenalectomized or intact animals.

The interrelationships of the thyroid were studied in the same manner. Thyroxine in maximal doses of 1 milligram did not induce fatty livers in intact fasted animals. Neither treatment with propylthiouracil nor surgical thyroidectomy caused an increase in liver fat by themselves; these procedures did not abolish the liver fat response to injected pituitary extracts.

No increase in liver fat was obtained with the following materials: desoxycorticosterone acetate, 5 mg. in sesame oil; stilbestrol, 5 mg. in sesame oil; epinephrine hydrochloride, .4 mg. (aqueous); regular insulin, .5 u.; testosterone acetate, 25 mg. in sesame oil, or; crystalline progesterone, 10 mg. in sesame oil.

Doses of choline chloride up to 2.5 mg. were shown to cause no

depression of fatty liver response to fat-mobilizing factor in accord with the observation of MacKay and Barnes (1938).

The effect of cold was studied by exposing the animals to an environmental temperature of 6°C. for seven hours. This caused a moderate increase in liver fat (averaging 7.34 per cent), as described by Leblond, Van Thoai, and Segal (1939). When fat-mobilizing factor was given during the period of cold exposure, the liver fat showed a normal response. When wire-bottom cages were used in the experiments which were conducted at room temperature, a variation in response was observed. This might also indicate some influence of exposure upon the effect.

DISCUSSION

Earlier investigators found the fat-mobilizing factor to be absent in the anterior pituitary (Coope and Chamberlain, 1925). These negative results might have been due to the use of preparations such as Antuitrin T-197, which has a very low potency of this factor. Best and Campbell (1936) showed fat-mobilizing effect to be present in the anterior pituitary.

Depression of fat mobilization in the rat by adrenalectomy, as described by Fry (1937), MacKay (1937) and others, seems to hold also in the mouse. The restoration by adrenal cortical hormone (and yeast) of the ability of phosphorus to cause fatty livers in adrenalectomized rats has been described by Verzar and Laszt (1936).

The lack of correlation of the fat-mobilizing potency with the adrenocorticotrophic activity of the preparations used, and the failure of large doses of cortical extracts to produce fatty livers in the intact mouse, indicate that the fat-mobilizing factor is not identical with adrenocorticotrophin, nor, by similar reasoning, with thyrotrophin. Likewise, the inability to correlate the fat-mobilizing factor with the potency of any of the other better known trophic hormones indicates that it is distinct from them. However, since adrenal cortical secretion is necessary for the immediate mobilization of fat, the adrenal must, in some way, facilitate the mobilization of depot fat. These findings might be associated in a general way with the known interplay of glycogen on body fat deposition (Wertheimer and Shapiro, 1948) and the glycogenic effect of adrenal cortical hormone. The possibility thus presents itself that the adrenal by its glycogenic function acts on immediate fat mobilization by priming the body fat depots for the action of fat-mobilizing factor, analogous to the preparation of the breasts by ovarian function for institution of lactation by pituitary extract (Stricker and Greuter, 1929).

Fat mobilization in response to stress as suggested by Leblond (1939), who considered it as a part of the adaptation syndrome of Selye, was not observed in these experiments. Substances which are known to cause profound adrenal stimulation, i.e. stilbestrol, insulin,

and epinephrine, were without effect in producing fatty livers, nor did adrenal cortical hormone alone cause the effect. Progesterone, testosterone, and stilbestrol were found not to be effectors in the mechanism of fat mobilization.

Recently Szego and White (1948) studied the effect of growth hormone (Wilhelmi, Fishman, and Russell, 1948) on liver fat. They found that adrenalectomy did not inhibit fat mobilization in 48-hour fasted male mice. This might indicate that with prolonged fasting another mechanism of fat mobilization, independent of the adrenals, could be operative. The absence of depot fat in adrenalectomized animals, as mentioned by Wertheimer (1948), would support the view that there is some utilization of body fat in such animals.

The lack of thyroid effect on immediate fat mobilization would support the view of Himsworth (1947) that the effect of the thyroid in production of fatty liver is nutritional in origin.

The inability of adrenal cortical extract to cause increased fat mobilization by fat-mobilizing factor in the intact animal indicates that the effect of the adrenals on fat mobilization is adequate for maximal stimulation in the animal with normally functioning adrenals. The slight depression of liver fat observed in these animals might be due to increased metabolism of fats in the livers of such animals with subsequent rapid utilization.

SUMMARY

The effect of various anterior pituitary preparations on the liver fat concentration of fasted mice has been investigated using a simplified method for the determination of total fat in the liver. The deposition of fat in the liver appeared to be caused by a separate substance, termed the fat-mobilizing factor which could not be identified with any one of the well-recognized anterior pituitary hormones. Though the adrenals are essential to the action of this factor, the effect is not due to adrenocorticotrophin nor is the effect mediated by the adrenal cortex.

ACKNOWLEDGMENTS

The author greatly appreciates the helpful guidance of Dr. E. B. Astwood in this problem.

We are indebted to Dr. David Klein of the Wilson Laboratories for the acetone-dried pituitary powders, to Dr. D. A. McGinty of Parke-Davis and Company for samples of Antuitrin T, and to Dr. John R. Mote of the Armour Laboratories for preparations of corticotrophin, thyrotrophin, and growth hormone. The Lipo-Adrenal Cortex was kindly supplied by Dr. H. F. Hailman of the Upjohn Company.

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ASCORBIC ACID IN THE PITUITARY OF THE RAT[1]

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THE RECENT work of Long (1947a, b) and Sayers and Sayers (1948) has demonstrated that exposure to stress or injection of ACTH results in the depletion of approximately 40–50% of the ascorbic acid normally present in the adrenal cortex. While the specific role of this substance in the formation and release of the adrenal corticoids is not yet known, it has been generally accepted that the loss of this vitamin is associated with the release of the cortical steroids. Thus it has been postulated that the changes in the concentration of ascorbic acid in the adrenal cortex may be used as an index of adrenal cortical activity (Long, 1947b).

It has also been reported that the hypophysis contains a high concentration of ascorbic acid (Giroud, 1938; King, 1939) and that a decrease in ascorbic acid content occurs in the pituitary of the female rabbit following copulation (Pincus, 1947). These reports led us to examine the vitamin C concentration of the pituitary gland of the rat and rabbit after subjecting the animals to various stimuli. It was felt that if changes were found in the vitamin C concentration of the pituitary, these alterations could be used as an index of activity in the hypophysis.

Secretion of the pituitary hormone was modified by several different procedures. Rabbits were mated or stimulated cervically with a faradic current of five volts to effect the release of gonadotropins and killed one hour later. Rats were subjected to (a) thiouracil[2] treatment in order to increase the thyrotropic activity of the pituitary gland, (b) electrical stimulation of the cervix during estrus to produce release of gonadotropins and (c) electrical stimulation of the vagus to release posterior pituitary hormone. The rate of secretion of ACTH was increased by (a) adrenalectomy, (b) exposure to cold (3 ± 1°C.) for one and twelve hours, and (c) intraperitoneal injection of epinephrine and histamine diphosphate. In the latter instance, the animals were killed 30 minutes after injection. Inhibition of ACTH secretion

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[2] Thiouracil was obtained through the courtesy of Dr. Stanton Hardy, American Cyanamide Company, Lederle Laboratories Division, Pearl River, New York.

was attempted by injecting cortin[3] and ACTH.[4] Normal animals of appropriate age and weight were used as controls.

All animals were killed by a sharp blow on the head. The pituitary gland was removed in less than 30 seconds, weighed and triturated in 6% trichloroacetic acid. Ascorbic acid content was determined according to the method of Roe and Keuther (1943).

Examination of the data (Table 1) shows that the amount of

TABLE 1. CONCENTRATION OF ASCORBIC ACID IN THE PITUITARY OF THE NORMAL AND TREATED RAT

No. of rats	Sex	Body wt. grams	Treatment	Pituitary weight mgm.	Ascorbic acid mgm. %
19	♀	81	—	3.9	112
15	♀	171	—	8.9	131
21	♀	216	—	12.2	145
7	♀	223	Cold—1 hour	13.3	145
4	♀	210	Cold—12 hours	13.5	140
16	♀	173	Adren. 24 hours	9.5	132
7	♀	184	Adren. 72 hours	9.8	135
8	♀	201	Histamine 10 mgm./100 gr. body wt.	11.5	143
9	♀	212	Epinephrine 0.2 mgm./100 gr. body wt.	11.8	140
4	♀	236	Cortin 10 dog units daily/6 days	14.9	157
5	♀	223	Cortin 10 dog units daily/11 days	13.4	145
3	♀	197	ACTH 2 mgm. daily/6 days	9.5	148
10	♀	196	Thiouracil 0.1% for 12 weeks	12.1	156
6	♀	220	Electrical stimulation of the cervix	13.4	143
16	♀	205	Electrical stimulation of the vagus	10.4	141
16	♂	218	—	7.4	139
17	♂	226	Electrical stimulation of the vagus	7.0	128

vitamin C in the hypophysis increases with age. It was found that the pituitary gland of the young female rat weighing 81 grams contained 112 mgm.% of ascorbic acid while in older rats weighing 171 and 216 grams, the concentration increased to 131 mgm.% and 145 mgm.% respectively. However, under the experimental conditions designed to alter the rate of secretion of ACTH, TSH, gonadotropins and posterior pituitary factor, no significant changes were found in the concentration of ascorbic acid in the pituitary gland.

Ascorbic acid determinations were also carried out on the pituitaries of female rabbits. Three rabbits killed one hour after mating showed a concentration of 113 mgm.% vitamin C in the pituitary gland while an average of 114 mgm.% was found in the pituitary gland of 5 control animals. In addition, no significant change was

[3] Cortin was obtained through the courtesy of Parke-Davis Company.

[4] ACTH was obtained through the courtesy of Armour & Company, Chicago, Illinois.

noted in ascorbic acid level of the hypophysis of three rabbits after electrical stimulation of the uterine cervix.

SUMMARY AND CONCLUSIONS

The normal concentration of ascorbic acid in the pituitary glands of adult rats remains constant. Experiments designed to produce changes in the content or production of TSH, ACTH, gonadotropins and posterior pituitary hormone failed to change significantly the concentration of ascorbic acid. No changes from normal were noted in the ascorbic acid concentration of the pituitaries of rabbits following mating or electrical stimulation of the uterine cervix.

An increase in ascorbic acid concentration of the pituitary gland was observed with increase in age of the rat.

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CYTOTOXIC ACTION OF HORMONES OF THE ADRENAL CORTEX ACCORDING TO THE METHOD OF UNSTAINED CELL COUNTS

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THE REACTIONS of cells to x-rays (Schrek, 1946) and nitrogen mustard (Schrek, 1947) have been studied previously by the method of unstained cell counts. This method has been used in this work to determine the effect of extracts and hormones of the adrenal cortex on cells of the thymus and of the bone marrow.

METHODS

The thymic gland or the bone marrow from the femurs of a rabbit was removed and placed in a small amount of Ringer's solution containing 5% isotonic phosphate buffer pH 7.6. The organs were chopped up with a fine pair of scissors and were then passed through a Seitz filter equipped with an 80 mesh Monel metal wire gauze. The cellular suspension thus obtained was washed once and resuspended in a mixture of equal parts of phosphate-Ringer's solution and rabbit serum.

The suspension was distributed into small test tubes in 0.4 ml. amounts. The reagent in various dilutions was added in 0.1 ml. amounts. The test tubes with the mixtures were placed in a water bath at 37°C. and shaken at the rate of 150 times per minute.

The reagents consisted of commercial extracts of the adrenal cortex and also preparations contributed by several laboratories. The commercial preparations included Lipo-Adrenal Cortex of the Upjohn Company and Eschatin of Parke, Davis and Company. Dr. W. J. Haines of the Upjohn Company generously contributed a dry extract of the adrenal cortex and the pure adrenal steroids, corticosterone and 17-hydroxycorticosterone. Adrenocorticotropic hormone of the pituitary gland was provided through the courtesy of Dr. C. H. Li and Dr. John C. Mote.

Dilutions of Lipo-Adrenal Cortex and of desoxycorticosterone were made with peanut oil, sesame oil or serum. Eschatin and the adrenocorticotropin were diluted with Ringer's solution.

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The pure adrenal cortical steroids and the extract of the adrenal cortex was dissolved in 95% ethyl alcohol, and diluted with distilled water so that the final concentration of alcohol was 1%. In some experiments the hormones or extracts were diluted with 95% alcohol, the dilutions were then distributed to test tubes and the alcohol evaporated in vacuo at room temperature.

To count the number of viable cells, 3.5 ml. of a solution of safranine 1:4000 in Tyrode's solution was added to the 0.5 ml. of suspension in a test tube. A drop of the mixture was placed in a hemocytometer and the unstained, stained and red blood cells were counted. The number of unstained cells per micromilliliter was then calculated.

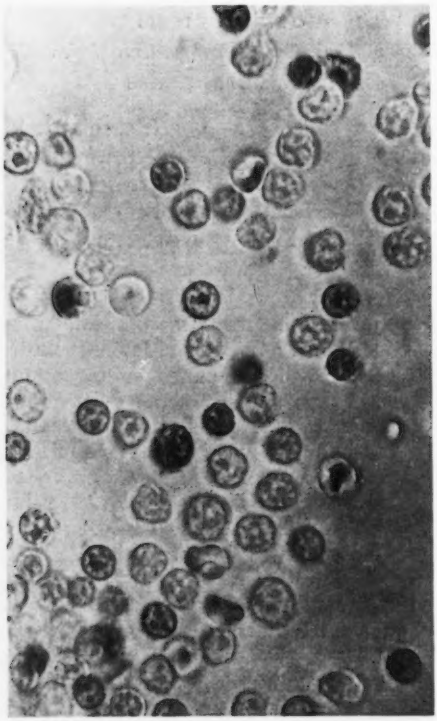

FIG. 1. Cells in a fresh suspension of a rabbit thymus. The suspension has been mixed with a safranine solution. Most of the cells are unstained, i.e. viable. A few cells are stained, i.e. dead. There is also an occasional erythrocyte. (×1000).

Effect of corticosterone on cells of the thymus. In a typical experiment the cells of a rabbit thymus were suspended in a medium consisting of homologous serum and phosphate-Ringer's solution. To a small quantity of the cellular suspension was added safranine, and a drop of the mixture was placed in a hemocytometer.

About 82% of the cells were (as in fig. 1) colorless, translucent

and round with fine granules throughout the cell. These cells which resisted staining with safranine may be considered as viable. The reasons for this assumption are discussed in a previous paper (Schrek, 1943).

About 14 per cent of the cells in the fresh suspension were stained red with the basophilic safranine as can be seen in fig. 1. The nuclei of these cells were deeply stained and had a thin nuclear membrane and fine chromatin granules. The ability of these cells to stain rapidly and intensely indicated that these cells were dead. They were pre-

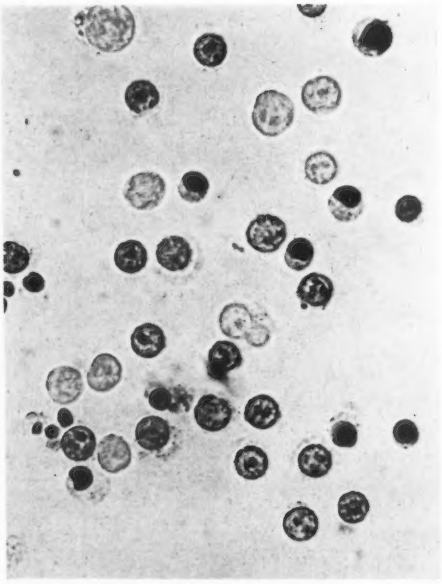

FIG. 2. Cells of rabbit thymus in a suspension incubated 24 hours at 37°C and then mixed with a safranine solution. About half of the cells are unstained. The stained cells have either pyknotic or leptochromatic nuclei. (×880).

sumably killed in the preparation of the suspension. It is to be noted that only under certain exceptional experimental conditions was there any difficulty in differentiating between the completely colorless unstained cells and the stained cells.

In addition there were a few red blood cells which were unstained, but which were characterized by their yellowish color, their sharp contour and their lack of granularity.

In this particular experiment, the suspension had 231.5 unstained, 38 stained and 11 red blood cells per micromilliliter (table 1).

The suspension was distributed to test tubes in 0.4 ml amounts and varying dilutions of corticosterone in 1% alcohol was added in

0.1 ml amounts. The mixtures were incubated at 37°C. At periodic intervals viable cell counts were made by adding safranine (3.5 ml) to the suspension in a test tube and counting the unstained cells in a hemocytometer.

Immediately after the addition of hormone in 3- to 30-microgram amounts no appreciable change in the viable cell counts could be observed. The steroid did not have any immediate perceptible effect on the number of viable cells in the suspension.

In the control suspension incubated for 21 hours at 37°C, the number of viable cells decreased from 231.5 to 101.5 which is 43.8% of the

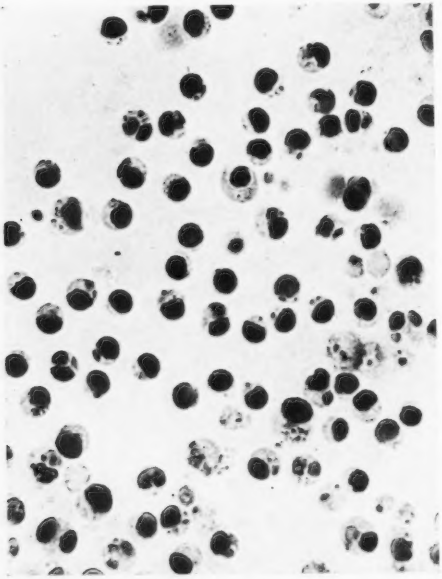

FIG. 3. Cells of a rabbit thymus in a suspension irradiated with 1000 *r* and incubated 24 hours at 37°C. Nearly all the cells have pyknotic nuclei. The same type of cells is obtained in suspensions treated with corticosterone. (×880).

original. In the suspension containing ethyl alcohol (0.2% final concentration), the same number of cells (101.0) survived. Evidently, the solvent used was not toxic to the cells.

Incubation of the suspension with 0.03 microgram of corticosterone had a small but definite toxic effect with the reduction of the viable cells to 71.5 as compared to 101.0 for the control. It appears that a perceptible effect was produced by 0.03 microgram in the presence of 116 million viable cells. Previous *in vitro* experiments have shown few reagents which are biologically effective in such minute quantities.

With a dosage of 0.3 microgram, fewer cells (only 61.3 or 26.5% of the original) survived the 21 hours of incubation. With 3 micrograms, the toxicity was increased further. But even this high dosage, 100 times the minimal, permitted 37.2 or 16.1% of the cells to survive the incubation. It seems that the hormone is only feebly toxic, in spite of the fact that it produces an effect even in low concentrations.

Additional observations at 27 and at 45 hours confirmed the findings obtained at 21 hours. Other experiments were conducted without the presence of alcohol and the results were substantially the same.

The table also shows the variations in the counts of viable cells. The average deviations for all counts in the table was 10% with vari-

TABLE 1. EFFECT OF CORTICOSTERONE ON SUSPENSION OF CELLS OF THE RABBIT THYMUS

Procedure: Test tubes with 0.2 ml. cellular suspension in phosphate Ringer's solution, 0.2 ml. rabbit serum, and 0.1 ml. corticosterone in 1% ethyl alcohol were incubated at 37°C. Counts were made after diluting the suspension with 3.5 ml. safranine in Tyrode's solution.

Hours of incubation	Final concentration		No. of viable cells per μml.				
	Alcohol %	Steroid μg.	Average count	Average deviation %	% of control: Based on original count**	% of control: Based on daily control***	Actual counts
0	0	0	231.5*	6.6	100		262-215-225-224
21	0	0	101.5	9.1	43.8		103-83-105-115
	0.2	0	101.0	6.4	43.6	100.0	106-100-89-109
	0.2	3	37.2	2.0	16.1	36.9	36.5-38
	0.2	0.3	61.3	2.0	26.5	60.8	62.5-60
	0.2	0.03	71.5	2.1	30.9	70.9	73-70
	0.2	0.01	101.0	17.8	43.6	100.0	83-119
45	0	0	26.5	18.9	11.4		23-36-27-20
	0.2	0	46.0	25.0	19.9	100.0	55.5-59.5-35.5-33.5
	0.2	3	5.4	3.7	2.3	11.6	4.8-5.6
	0.2	0.3	6.5	1.5	2.8	14.1	6.6-6.4
	0.2	0.03	29.3	16.0	12.7	63.8	24.5-34
	0.2	0.01	32.3	8.4	14.0	70.4	29.5-35

* The counts show the number of cells in the original suspension. The final concentration of viable cells in the 0.5 ml. mixture was 92.6 cells per μml. or 92,600 per cu. mm. The total number of viable cells in the mixture was 116×10^6.

** This column shows the percentages of viable cells surviving after incubation and treatment with the various reagents. These percentages are used as ordinates in graphs similar to fig. 4.

*** The differences between these percentages from 100% represent the % effect and is used in graphs such as fig. 6.

ations from 6.6% for the original suspension to 25% for some of the other counts. These deviations indicate a fairly high experimental error. Higher accuracy in the titration of the hormone would require a larger number of counts than was done in the present experiment.

Associated with the decrease in the number of viable cells was a corresponding increase in the stained cells. The control suspensions which were incubated 21 or more hours had many stained cells with granular, apparently well preserved nuclei (fig. 2). These cells were similar to those seen in the original suspension (fig. 1). Most of the stained cells, however, had pyknotic nuclei which were shrunken, round or oval and stained deeply and uniformly without any visible

structure (fig. 2). In some cells the pyknotic nucleus was fragmented into two or more parts.

Apparently the cells, in the absence of any added reagent, undergo on incubation two distinct types of degeneration or cell death—one leading to granular nuclei and the other resulting in pyknosis and fragmentation. From other experiments, it appears that the process leading to pyknosis represents the normal physiologic aging and death of the lymphocytes. Cellular death resulting in the granular nucleus appears to be due to extraneous, non-physiologic factors and can be obtained with such reagents as acid, low temperatures, and fixatives.

In the incubated suspensions treated with corticosterone, nearly all the stained cells had pyknotic nuclei as in fig. 3. A few stained cells with granular nuclei were probably those which were present in the original suspension. It appeared then that cells dying as a result of or in the presence of corticosterone are similar in morphologic appearance to cells dying spontaneously.

The question arises whether corticosterone acts by causing the early death of the cells or by inhibiting the growth of the cells in the suspension. To study this question, it is necessary to examine more closely the unstained cells in the suspensions.

To determine the morphology and the mitotic activity of the unstained viable cells, smears were made of the original and the incubated suspensions. The smears were then stained by hematoxylin and eosin and by Feulgen's stain. After 18 hours of incubation of the control suspension most of the cells appeared to be normal as far as the morphologic appearance of the nucleus. An occasional cell (about 1 in 500) showed a mitotic figure (see fig. 6 in a previous paper, Schrek, 1947). This finding suggests that there was some cell division in the suspension. That this tendency to cellular growth was low was indicated by the observations that at no time during the incubation was there an increase in the number of unstained cells and that there was no appreciable increase in the total number of cells in the suspension. It appears then that under the present experimental conditions, the cells in the control suspension are in a state of survival rather than in a state of growth.

The findings lead to the tentative conclusion that corticosterone decreased the survival period of the cells in the suspension.

Titration of 17-hydroxycorticosterone. The present experiments were designed primarily to determine whether the steroids of the adrenal cortex had any effect on lymphocytes in vitro. The data obtained in an experiment such as in table 1 can, however, be analyzed mathematically in order to determine the quantitative relationship between the dosage of the reagent and the reaction of the cells.[1]

The method of titration may be illustrated using the data obtained for another adrenal steroid, 17-hydroxycorticosterone. A table similar to table 1 was first prepared. Then survival curves of the cells

in control and treated suspensions were drawn. On logarithmic-probability (Schrek, 1941) or probit graph paper the survival curves may be represented by straight lines as can be seen in fig. 4.

The intersection of the rectilinear survival curve with the 50% line gives a statistical constant termed the 50% survival time. It is seen that the 50% survival time is 28 hours for the control cells, 19 hours for cells treated with 0.01 microgram of 17-hydroxycorticosterone and 10.8 hours for 0.3 microgram.

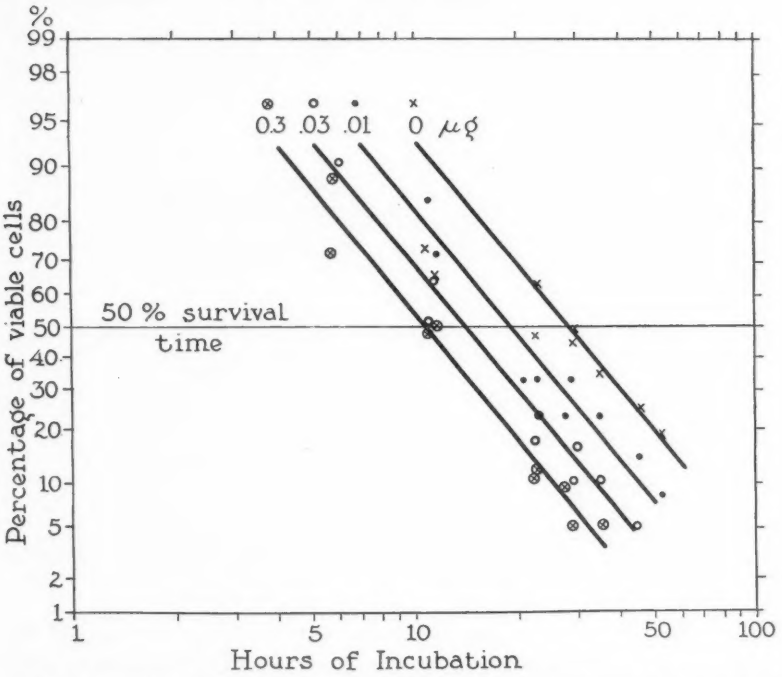

FIG. 4. Survival curves of thymic cells treated with corticosterone and incubated at 37°C. The ordinates represent the percentages of viable cells where 100 per cent is the number of viable cells in the fresh suspension. By use of probit paper, the survival curves can be represented as straight lines, and the 50 per cent survival time can be readily determined.

The 50% survival time is a measure of the reaction of the cells to both the reagent and the experimental conditions. To measure the susceptibility of the cells to the reagent alone, the 50% survival time of the treated cells was expressed, not in hours but as a percentage of the 18.3-hour survival time for the control suspension. By this procedure the 50% survival times were 67.8% for 0.01 and 38.6% for 0.3 microgram of 17-hydroxycorticosterone.

The next step in the analysis was to draw a dose-effect curve on

logarithmic probability paper using the dosage as abscissae. The "effect" is represented on the ordinates and is calculated by subtracting the 50% survival time in per cent from 100%. It should be noted in passing that it is more convenient to indicate on the ordinate scale percentages, which have biologic significance, instead of probits, which are only mathematical abstracts. The dose-effect curve for 17-hydroxycorticosterone is shown in fig. 5.

The dosage producing the 50% effect may be termed the median effective dose. In other words the median effective dose (MED) is

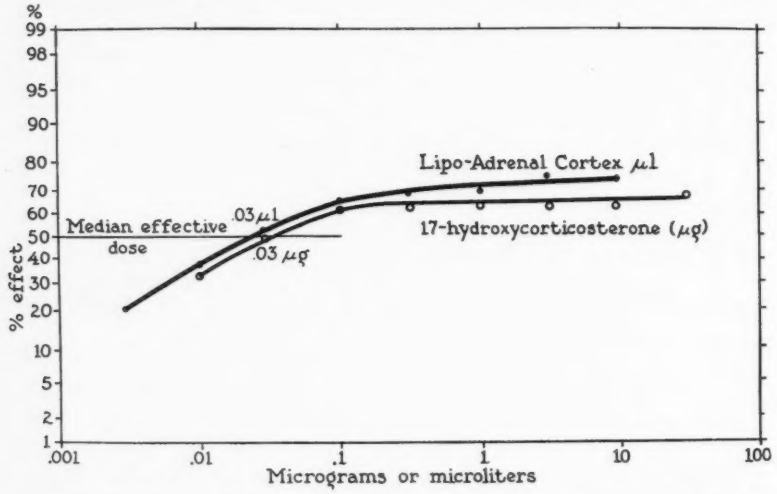

FIG. 5. The dose-effect curves on probit graph paper for thymic cells treated with Lipo-Adrenal Cortex and 17-hydroxycorticosterone. The dose-effect curves are curvilinear, show a maximum effect and permit the determination of a median effective dose.

that amount of reagent which reduces the 50% survival time of the cells by 50%. For 17-hydroxycorticosterone the MED was 0.03 microgram. The probable error of this constant cannot be determined by this graphical method, but it is undoubtedly very large due to an insufficient amount of data, and due to the low slope of the curve.

The dose-effect curve in fig. 5 is seen to be curvilinear and to approach a maximum effect of 70%. A dose as high as 30 micrograms (or about 1000 MED) failed to increase the effect beyond 70%. The minimum 50% survival time produced by the reagent was then about 9 hours.

The method described above for titration of 17-hydroxycorticosterone has, it is believed, theoretical significance and is particularly useful in studying a new compound. For practical purposes it is possible to simplify considerably both the experimental work and the calculations in determining the titration of the hormone. For this

method, it was necessary to consider only the viable cell counts at one particular time, as 21 hours. It is desirable to choose a standard observation period which should be approximately the 50% survival time of the cells studied (about 28 hours for cells of the rabbit thymus). The effect of the various amounts of reagent was then calculated using the viable cell count of the control (138 cells) as standard. A dose-effect curve was drawn on probit paper (fig. 6). This dose-effect curve is also curvilinear. Under these conditions a "standard effective dose" can be read from the graph and is found to be 0.02 microgram.

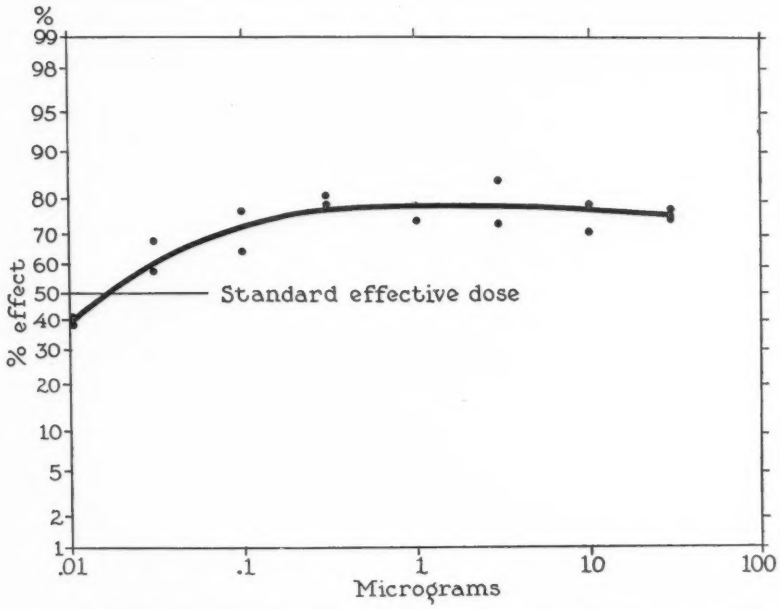

FIG. 6. Simplified dose-effect curve on probit graph paper for thymic cells treated with 17-hydroxycorticosterone and incubated for 22 hours at 37°C. As in Fig. 5, the dose-effect curve is curvilinear, has a maximum effect and permits the determination of a standard effective dose.

This compares with 0.03 microgram of the MED based on the 50% survival time.

Effect of hormones and steroids on lymphocytes. Dose-effect curves based on 50% survival time were also obtained for Lipo-Adrenal Cortex (fig. 5). This curve is also curvilinear and approaches a maximum of 70% effect. The MED is found to be 0.03 microliter or 0.001 rat unit according to the titration on the label of the commercial preparation. The dose-effect curves for Lipo-Adrenal Cortex and for 17-hydroxycorticosterone were similar in character.

TABLE 2. THE CYTOXICITY OF HORMONES AND EXTRACTS OF THE ADRENAL CORTEX AND OF NITROGEN MUSTARD AND X-RAYS TO CELLS OF THE RABBIT THYMUS. THE CYTOXICITY IS MEASURED BY THE MEDIAN EFFECTIVE DOSE OR THE DOSE THAT DECREASES THE 50% SURVIVAL TIME BY 50%

Reagent	Median effective dose	
	ml. or μg.	Units
17-Hydroxycorticosterone	0.03 μg.	
Corticosterone	1 μg.	
Lipo-Adrenal Cortex	0.00,003 ml.	0.001 rat units
Eschatin	0.00,03 ml.	0.015 dog units
Extract of Adrenal Cortex	2.35 μg.	0.002 rat units
Nitrogen mustard	0.46 μg.	
X-rays		140 roentgens

Eschatin, an aqueous extract of the adrenal cortex had an MED of 0.0003 ml or 0.015 dog unit (table 2).

Several experiments on dry extract of the adrenal cortex gave an MED of 2.35 micrograms (table 2) which was equivalent to 0.002 rat unit according to the titrations obtained by Dr. Haines using the muscle glycogen method.

The crystalline steroid, corticosterone, produced the same dose-effect curve as Lipo-Adrenal Cortex. The MED was about 1.0 micrograms. It should be noted that all of the titrations are only approximate.

TABLE 3. THE LOW CYTOTOXICITY OF HORMONES TO CELLS OF THE THYMUS

Reagent	Dose in mg. producing		
	Moderate or 50% effect (median effective dose)	Slight effect	No appreciable effect
Desoxycorticosterone Percorten (Ciba) Cortate (Shering)	—	0.5	0.05
Adrenocorticotropin	—	0.2	0.1
Diovocylin (Ciba)	—	—	0.5
Di-ethylstilbestrol (Lilly)	0.25	0.03	—
Progesteron	—	1.0	0.25
Testosterone	0.2	—	—
Epinephrine	—	0.2	0.1

Desoxycorticosterone, in contrast, produced no effect even in a dosage of 0.05 milligram (table 3). The addition of 0.5 milligram had a slight toxic action which was less than a 50% effect.

The adrenocorticotropic hormone provided by Dr. Li produced little if any effect with a dosage of 0.2 milligrams. Other hormones were tested including diovocylin, diethylstilbesterol, progesteron, testosterone and epinephrine. All of these compounds, even in fairly high dosage, had little or no effect on the survival of cells derived from the rabbit thymus. In one experiment both adrenocorticotropic

hormone and Lipo-Adrenal Cortex were added to the suspension. It was found that the pituitary hormone did not increase or inhibit the activity of the Lipo-Adrenal Cortex.

Dose-effect curves for nitrogen mustard and x-rays. The peculiarities of the dose-effect curves for the steroids can be better understood by comparison with the curves for other reagents, such as nitrogen mustard and x-rays (fig. 7). The dosage on the abscissae of the graph is expressed in terms of MED which is about 140 r for x-rays and 0.46

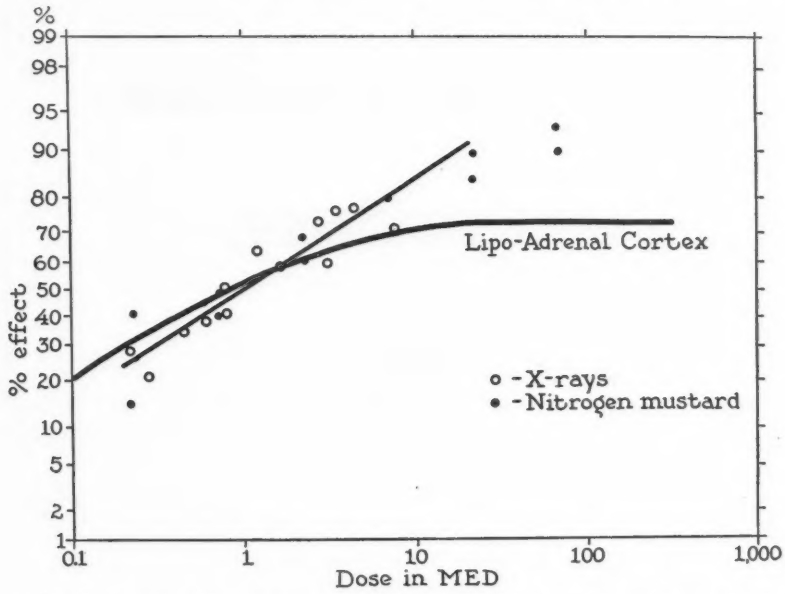

FIG. 7. A comparison between the dose-effect curve for Lipo-Adrenal Cortex, x-rays and nitrogen mustard. The dosage is given in terms of the median effective dose for each reagent. The dose-effect curves for x-rays and nitrogen mustard are identical, and differ from the dose-effect curve for Lipo-Adrenal Cortex in being linear and in not having a maximum effect.

microgram for nitrogen mustard. The figure shows that the dose effect curves for the two reagents are, to a considerable extent, linear and have approximately the same slope. About 93% effect was produced by 90 MED's of nitrogen mustard as compared to a 70% maximum effect by 300 MED's of Lipo-Adrenal Cortex.

From this comparison, it appears that the dose-effect curves of the adrenal steroids are characterized by the curvature, the 70% maximum effect and the low median effective dose. These three peculiarities in the curves seem dependent upon the nature of the toxic action produced by Lipo-Adrenal Cortex, Eschatin, corticosterone and 17-hydroxycorticosterone. From a teleologic point of view, it does

not seem strange that a hormone produces an effect with low concentrations and that this effect is mild even with high concentrations.

Reaction of cells of the bone marrow. Suspensions of cells of the bone marrow of rabbits were prepared and tested in the same way as the cells of the thymic gland. The viable cells in the suspensions of bone marrow were more difficult to count because of the necessity to differentiate between the unstained living cells and the unstained mature and immature red blood cells. It has been seen that for cells

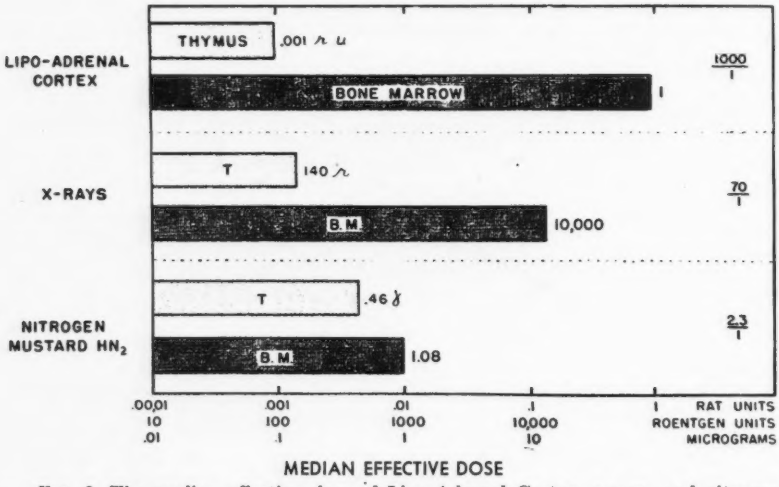

FIG. 8. The median effective dose of Lipo-Adrenal Cortex, x-rays, and nitrogen mustard for cells of the thymus and of bone marrow. The thymic cells were susceptible to the action of all three reagents as indicated by the low median effective dose. In contrast, the cells of the bone marrow were relatively resistant to the action of Lipo-Adrenal Cortex and x-rays as indicated by the large median effective dose. *r u* is rat units, *r* is roentgens and γ is micrograms.

of the thymus, the median effective dose of Lipo-Adrenal Cortex was .001 rat unit. In experiments with suspensions from the bone marrow, the median effective dose was found to be approximately 1 rat unit (fig. 8). The relative susceptibility of the two types of cells was 1000 to 1. The cells from the bone marrow were then almost completely resistant to the action of Lipo-Adrenal Cortex.

Previous work has shown that the cells of the bone marrow were also fairly resistant to x-rays. The median effective dose was 140 r for cells of the thymus and about 10,000 r for cells of the bone marrow. The ratio of susceptibilities was 70 to 1.

With nitrogen mustard the median effective dose was 0.46 microgram for cells of the thymus and 1.08 for cells of the bone marrow with a ratio of only 2.3 to 1. It seems then from these experiments that cells of the bone marrow are resistant to the action of Lipo-Adrenal Cortex and to x-rays, but not to nitrogen mustard.

Effect of temperature of incubation. It has been seen that extracts of the adrenal cortex have a delayed cytocidal action on cells from the thymus. The mechanism of this reaction was studied by determining the effect of the temperature of incubation on the reaction of cells and reagents. Mixtures of suspensions and Lipo-Adrenal Cortex were prepared at room temperature, and were then incubated at

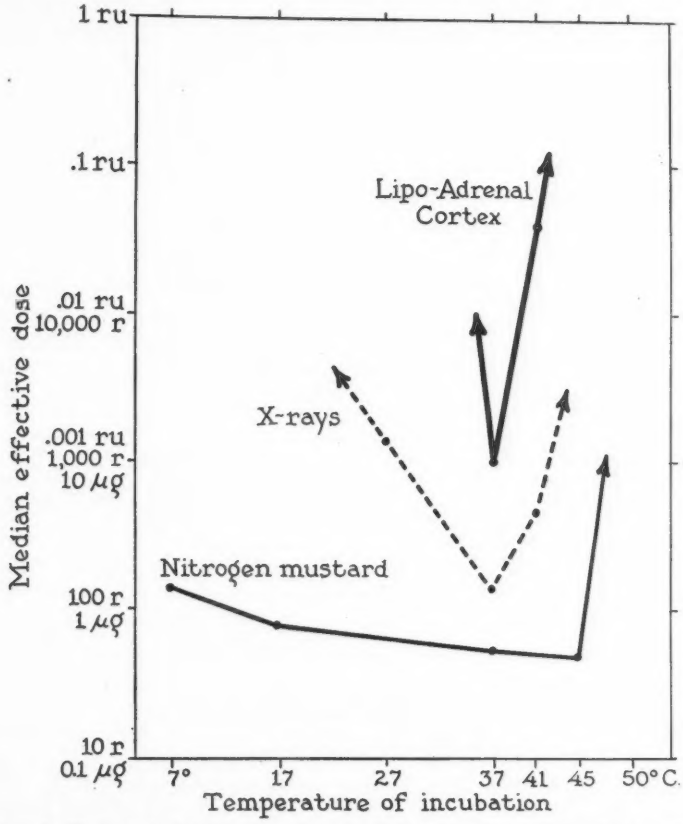

FIG. 9. The effect of temperature of incubation on the median effective dose of Lipo-Adrenal Cortex, x-rays, and nitrogen mustard for thymic cells. Nitrogen mustard was toxic to the cells at a wide temperature range of 7 to 45°C whereas Lipo-Adrenal Cortex and x-rays were toxic only in a narrow temperature range.

varying temperatures. Counts of the viable cells were made and survival curves and dose-effect curves were prepared. The susceptibility of the cells to the reagent at any particular temperature was measured by the median effective dose. At 37°, the MED was approximately .001 rat unit of Lipo-Adrenal Cortex (fig. 9). The maximum effect produced by large doses of reagents at 37° was 70%. On incubation at

27°C. the maximum effect obtained was less then 50% so that one has to consider the MED is greater than the maximum dose tested or 1 rat unit. At 45°, the reagent had no appreciable effect on the survival of the cells. It would seem then that the Lipo-Adrenal Cortex affected the survival of the lymphocytes only when the cells were incubated at approximately physiologic temperature.

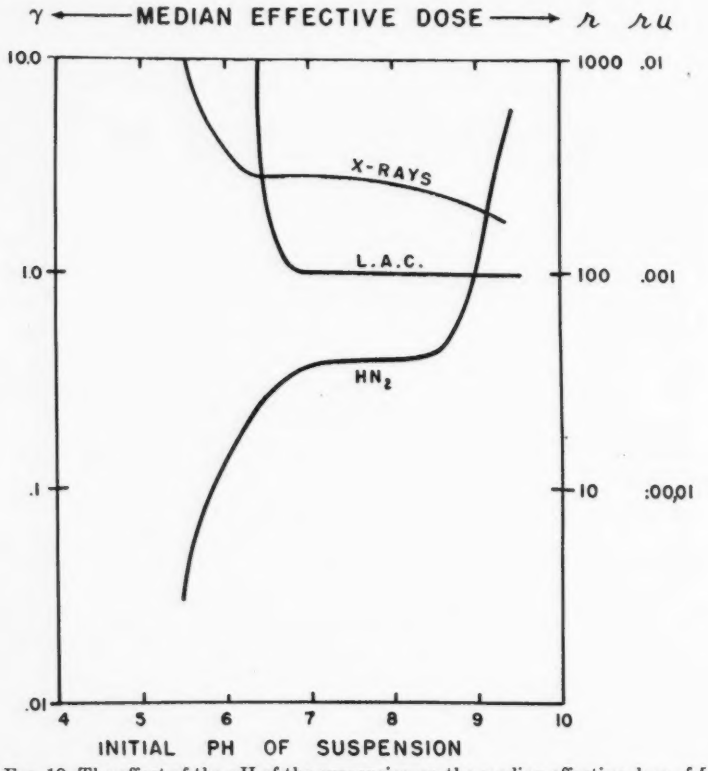

FIG. 10. The effect of the pH of the suspension on the median effective dose of Lipo-Adrenal Cortex, x-rays and nitrogen mustard for thymic cells. Acid reduced the susceptibility of the cells to the action of Lipo-Adrenal Cortex and x-rays but increased their susceptibility to nitrogen mustard.

It is of interest that x-rays also had a narrow temperature range of effectiveness as can be seen in the figure. At 27° and 41° the median effective dose of x-rays was less than at 37°, but at 17° and 45° x-rays had no appreciable effect on the survival of the cells.

Similar studies have been done with nitrogen mustard as a reagent. This reagent had a median effective dose of 0.46 microgram at 37°C. Varying the temperature between 17° and 45° had but little effect on the MED of nitrogen mustard. At 50°, however, the cells were resist-

ant to the action of nitrogen mustard and, in fact, the treated cells survived longer than the untreated ones. Nitrogen mustard then was toxic to cells over a wide range of temperature whereas x-rays and Lipo-Adrenal Cortex was effective only in a narrow range.

Effect of pH. A few experiments were done on the susceptibility of cells to Lipo-Adrenal Cortex, x-rays and nitrogen mustard, at different pHs. The pH of the suspension was altered by addition of hydrochloric acid or sodium hydroxide before Lipo-Adrenal Cortex or nitrogen mustard was added. In the case of the suspensions treated with x-rays, the cells were first radiated and then the pH of the suspension was adjusted. The susceptibility of the cells as measured by the median effective dose are represented graphically in fig. 10. It is seen that the cells in a fluid at a pH of approximately 6 were resistant to the action of both Lipo-Adrenal Cortex and x-rays, but they were relatively more susceptible to the action of nitrogen mustard. At pH of 9, the cells were approximately as sensitive to the action of x-rays and Lipo-Adrenal Cortex as at pH 7, but the toxicity of nitrogen mustard appeared to be increased in alkaline medium. There seems then to be a similarity in the effect of x-rays and Lipo-Adrenal Cortex on cells in acid and alkaline media.

DISCUSSION

Toxicity of hormones of the adrenal cortex. The present experiments showed that extracts and pure hormones of the adrenal cortex had an appreciable effect on cells of the rabbit thymus in vitro. This effect was evidenced by the increase in the number of cells which stained readily with safranine and by a decrease in the number of safranine-resistant cells. The findings were interpreted as indicating that the extracts and hormones caused a relative increase in the number of dead cells in the suspension and a decrease in the number of living cells.

The observations could be interpreted in three ways: 1) the steroids have a direct cytocidal action on the lymphocytes; 2) they accelerate the normal rate of physiologic aging and death of the cells; or 3) they inhibit the mitotic activity and the growth of the lymphocytes in the suspension, but do not affect the death rate of the cells. No direct evidence has been obtained to support conclusively any one of these hypotheses. It seems, however, that the most plausible explanation of the observations is the second hypothesis that the steroids accelerate the aging and death of the cells.

The experimental findings indicate that the action of the steroids on lymphocytes had certain characteristics. First, the reagents tested had no immediate effect on the cells. Secondly, minute quantities of the steroids produced a detectable, delayed effect. Third, large doses had no greater effect than moderate doses. The maximum effect produced by the reagents was a relatively mild one. Finally the steroids

had no preceptible effect on intact dead lymphocytes in the suspension, nor on living cells of the bone marrow. These observations may aid in interpreting some of the reports in the literature.

In vivo, it has been shown by Dougherty and White (1945) and Selye (1947) that extracts of the adrenal cortex cause the necrosis of lymphoid tissue and ultimately the decrease in the weights of the lymphoid organs. The present in vitro findings are in accord with this in vivo observation of necrosis in lymphoid tissue. In vivo, the necrotic tissue would, of course, be rapidly absorbed whereas in vitro the dead cells remained intact.

The present findings are also in accord with the in vitro work of Heilman (1945). She reports that compound E, in concentrations of 1.25 micrograms per milliliter caused a moderate but significant inhibition of the migration of lymphocytes from explants of a rabbit lymph node in tissue culture. This inhibition was associated with an increased rate of degeneration of small and medium-sized lymphocytes in the migration zone. Increasing the concentration of the reagent failed to increase the degree of inhibition. In addition, Heilman (1945) found that the pure adrenal hormones had no inhibiting effect on macrophages in tissue culture. These findings are in agreement with the present observations of 1) the deleterious effect of pure adrenal steroids on rabbit lymphocytes, 2) the low dosage required for this effect, 3) the failure of larger doses to increase the effect and 4) the failure of the reagent to act on cells of the bone marrow.

Robertson (1948) observed that extracts of adrenal cortex failed to cause lysis of living lymphocytes in vitro. This finding is in agreement with the present observations that the steroids decreased the number of living and increased the number of dead cells, but had no appreciable effect on the total number of cells in the suspension.

Hechter and Stone (1948) found that extracts of adrenal cortex caused a rapid decrease in the number of lymphocytes in suspension. The apparent disagreement of this finding with the present observations may be attributed to differences in the experimental procedures.

It may not be amiss to emphasize that the observed toxic action of the steroids on lymphocytes is a relatively mild one. The method of testing has to be quite sensitive in order to demonstrate this activity of the hormones.

The mechanism of action of adrenal cortex hormones. The present findings throw some light on the problem of the mechanism. In the first place the end result of the action of the hormones on lymphocytes is a cell with a pyknotic nucleus. The same end result is obtained in cells undergoing spontaneous death and in cells killed with x-rays. Other toxic agents such as alcohol, heat, cold, formaldehyde and high concentrations of nitrogen mustard killed cells without producing a pyknotic nucleus.

There are several similarities in the reaction of the cells to x-rays

and to adrenal cortex hormones. The reagents were similar in that 1) they killed cells with the production of a pyknotic nucleus, 2) they were effective only when the cells were incubated in a narrow temperature range, 3) they were inhibited in the presence of acid, and 4) they were toxic to lymphocytes, but not to cells of bone marrow. The similarities would suggest that both reagents affect the same physiologic process within the cell. It would seem, furthermore, that both reagents accelerate the normal physiologic degeneration and death of the cells.

Current hypotheses suggest that x-rays affect the metabolism of thymonucleoprotein. There is then the possibility that the hormones of the adrenal cortex also affect the metabolism of this protein in lymphocytes.

In view of the similarity of the action of x-rays and extracts of adrenal cortex in lymphocytes, the question may be raised whether the two reagents affect other cells in similar manner. One action of x-rays is inhibition of growth. Recent studies have shown that hormones of the adrenal cortex can inhibit growth generally and locally. Another property of x-rays is inhibition of mitosis. Further investigation would be in order to determine whether adrenal cortex hormones have a direct effect on cells in mitotic division.

One difference in the behavior of cells treated with x-rays and with adrenal cortex hormones was observed in the present studies. The two reagents produced different dose-effect curves. For x-rays, the dose-effect curve was linear whereas for the hormones it was curvilinear. The curvilinear dose-effect curve for the hormones might be due to secondary factors such as permeability of the cell to the hormones.

SUMMARY

The following reagents had a delayed but not an immediate in vitro cytotoxic action on cells from the thymic gland of rabbits: Lipo-Adrenal Cortex, Eschatin, extracts of the adrenal cortex, corticosterone and 17-hydroxycorticosterone. These reagents were active in low concentrations. Increasing the dose of the reagent did not increase markedly the effect produced.

The Lipo-Adrenal Cortex was active when cells were incubated at 37° or 41°, but not at 27° or 45°.

Acid at a pH of 6 inhibited the action of Lipo-Adrenal Cortex.

No appreciable effect was observed with adrenocorticotropic hormone, desoxycorticosterone and with various male and female sex hormones.

Lipo-Adrenal Cortex had no appreciable effect on cells of the bone marrow of rabbits.

There are several points of similarity in the action of x-rays and of Lipo-Adrenal Cortex on cells of the thymic gland.

The hypothesis is proposed that the extracts of the adrenal gland accelerate the normal degeneration and death of lymphocytes.

ACKNOWLEDGMENTS

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ADRENOTROPHIC ACTIVITY OF HUMAN BLOOD[1]

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THE PRESENCE of adrenocorticotrophic hormone, or of a substance or substances possessing adrenotrophic activity, has not been convincingly demonstrated in human urine (Locke, Albert and Kepler, unpublished). We have therefore turned our attention to the blood, in the hope that adrenotrophic material may be present in a concentration sufficiently high to be measured by current assay procedures. A review of the literature pertinent to this subject is summarized in table 1. It appears that the results obtained are conflicting, and that much of this confusion has resulted from the use of test animals with intact pituitaries. Such a procedure does not, of course, permit specific detection of adrenotrophic activity in the material tested. Thus, there is considerable doubt as to the validity of all results obtained with human blood, with the sole exception of those obtained by Cooke, Graetzer and Reiss (1948).

The increasing emphasis on the study of pituitary-adrenal relationships makes evident the value of investigative work which may clarify this interrelationship. To avoid as far as possible the usual pitfalls encountered in studies of this type (Albert, 1949), we have investigated the problem of detection and assay of adrenotrophic hormone in human blood with the following points of emphasis: (1) the use of a sensitive and specific assay method so as to avoid spurious results, (2) the use of fresh untreated serum so as to obviate the use of empirical methods of concentration and purification, and (3) the use of blood from patients with adrenal disorders, situations in which extreme ranges of adrenotrophin might conceivably be encountered.

MATERIAL AND METHODS

The method of assay for adrenotrophic activity was that described by Sayers, Sayers and Woodbury (1948). Two minor modifications of this pro-

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TABLE 1. TESTS FOR ADRENOTROPHIC ACTIVITY IN BLOOD (SERUM OR PLASMA)

Reference	Source of blood*	Preparation	Serum volume per animal, ml.	Assay method	Adrenotrophic activity
Jores (1935)	Cushing's syndrome	Deproteinized, concentrated serum	≈12	Increase in adrenal wt., and histologic change in intact mice	Yes
Sievert (1938)	Hypertensives 1. Benign 2. Malignant 3. Nephritic	Plasma ultrafiltrate	3–6	Disappearance of sudanophobe zone in intact mice	 1. Yes 2. Yes 3. No
Paschkis (1940)	1. Cushing's syndrome 2. Adrenal cortical tumor 3. Hypertensives	Not stated	Not stated	Method of Jores (above)	1. Yes 2. No 3. No
Rakoff, Cantarow and Paschkis (1941)	Cushing's syndrome	Not stated	Not stated	Method of Jores	Yes
Cabeza (1942)	Diabetics	Deproteinized, heated and concentrated serum	≈20	Increase in adrenal wt., and histologic change in intact mice	Yes
Bartelheimer and Cabeza (1942)	1. Insulin hypoglycemia 2. Cushing's syndrome 3. Morgagni's syndrome 4. Acromegaly 5. Diabetics	Deproteinized, heated and concentrated serum	≈20	Method of Cabeza (above)	 1. Yes 2. Yes 3. Yes 4. Yes 5. Yes
Golla and Reiss (1942)	Pregnant (mare)	1. Acetone dried serum 2. Boiled serum	Not stated	Increase in adrenal wt. in hypophysectomized rats	1. Yes 2. Yes
Faber (1945)	Adrenalectomized (rat)	Untreated serum	10	Increase in adrenal wt. and histologic change in hypophysectomized rats	No
Cooke, Graetzer and Reiss (1948)	Normal	Acetone precipitate of plasma	5–10	Ascorbic acid depletion in adrenals of hypophysectomized rats	Yes

* Except when otherwise indicated, all tests were performed with human blood.

cedure were employed: (1) hypophysectomized male rats of the Wistar strain were used as the test animals instead of the Sprague-Dawley strain used by Sayers and associates, and (2) the serum was administered via the inferior vena cava at the level of the right renal vein instead of via the tail vein.

Blood was obtained from normal subjects, patients with adrenal cortical hyperfunction and patients with adrenal cortical insufficiency by venipuncture in the antecubital fossa. In a few cases, blood was obtained from the external jugular vein. It was allowed to stand for two to four hours and then centrifuged to recover the serum. The serum was refrigerated at 4°C. until

TABLE 2. ASCORBIC ACID CONCENTRATIONS IN THE ADRENALS OF HYPOPHYSECTOMIZED RATS GIVEN PHYSIOLOGIC SALINE SOLUTION

No. of test	Volume of saline, ml.	Concentration of ascorbic acid, mg./100 gm. tissue		
		Left adrenal (before saline)	Right adrenal (1 hr. after saline)	Left–Right (L–R)
1	2.5	480.9	494.0	−13.1
2	3.0	457.1	467.6	−10.5
3	2.5	464.7	443.3	21.4
4	3.0	389.3	378.3	11.0
5	2.5	458.8	486.7	−27.9
6	2.5	496.1	516.1	−20.0
7	2.5	473.7	484.8	−11.1
8	2.5	516.2	512.2	4.0
9	2.5	507.8	522.5	−14.7
10	2.5	593.6	593.2	0.4
11	2.5	507.3	531.4	−24.1
12	2.5	488.7	491.6	− 2.9

Mean = -7.3 ± 4.2*

* Standard error of mean (Sem).

$$\text{Sem} = \frac{\text{S.D.}}{\sqrt{n}}$$

used, in most instances a period of about twenty hours. As a rule, 2.0 to 3.0 ml. of serum were injected into each rat, 3 rats being employed for each sample of serum. The determination of the ascorbic acid concentration of both adrenal glands was carried out according to the method described by Roe and Kuether (1943), adapted for use with tissue. Readings were made in a Coleman, Jr. spectrophotometer at a wavelength of 510λ, and the ascorbic acid concentrations were reported in terms of milligrams of ascorbic acid per 100 gm. of fresh adrenal tissue.

Adrenotrophic activity was expressed as the difference in ascorbic acid concentration between the left adrenal, removed as a control before injection, and the right adrenal, removed one hour after the injection of the serum to be tested. This procedure is hereafter designated as "left minus right," or "L–R."

In order to obtain control values with which the experimental values could be compared, the effect of solutions containing no adrenotrophic activity on the adrenal ascorbic acid concentration was investigated. After the removal of the left adrenal, 2.5 to 3.0 ml. of 0.9 per cent saline solution were administered to 12 hypophysectomized rats (table 2) according to the previ-

ously described procedure. The concentration of ascorbic acid in the right gland one hour after administration of the solution was subtracted from the concentration of ascorbic acid in the left gland (L–R). In the group of 12 animals the mean concentration of ascorbic acid in the right gland was higher than in the left by 7.3 ± 4.2 mg. per 100 gm. of fresh tissue, or a mean L-R of -7.3 ± 4.2. Although in principle these results are in agreement with those of Sayers, Sayers and Woodbury, our variation appears to be much greater than that encountered by these investigators. Our variability would indicate that no single L–R value less than 20 mg. can be accepted as definitely indicative of the presence of adrenotrophic activity, at least under the test conditions used by us.

RESULTS

Adrenotrophic Activity of Normal Serum.—Serum obtained from 3 normal women and 4 normal men, whose ages ranged from 18 to 45 years, was assayed for adrenotrophic activity, as shown in table 3. Only 2 of the 7 serums caused the ascorbic acid concentration of the right gland to fall below that of the left, so that the mean L–R was -4.4 ± 5.9 mg. However, serum obtained from the external jugular vein of 1 normal subject and administered to 3 rats in a dose of 1.0 ml. gave a mean L–R of 34.9 mg. The latter data are not included in table 3.

Adrenotrophic Activity of Serum in Patients Having Adrenal Cortical Hyperfunction.—Serum was obtained from 6 patients with adrenal cortical hyperfunction, proved at surgical exploration to be due

TABLE 3. ASSAY OF NORMAL SERUM* FOR ADRENOTROPHIC ACTIVITY

Patient, sex and age, years	Reduction of adrenal ascorbic acid, left gland–right gland (L–R), mg./100 gm. fresh adrenal tissue	
	Individual values	Mean
1 Woman 21	−11.4, −22.1, 21.1	− 4.1
2 Woman 45	−24.0, −13.8	−18.9
3 Woman 18	− 2.4, −53.5	−27.9
4 Man 38	37.9, 15.2, 0.4	17.8
5 Man 29	12.1, 2.6, 17.1	10.6
6 Man 28	−11.4, −22.9, 14.5	− 6.6
7 Man 33	21.3, − 0.9, −24.8	− 1.5
	Group mean = -4.4 ± 5.9	

* 2.5 ml. per test animal.

TABLE 4. ASSAY OF ADRENOTROPHIC ACTIVITY IN THE SERUM OF PATIENTS HAVING ADRENAL CORTICAL HYPERFUNCTION

Patient, sex and age, years	Diagnosis	Serum volume per test animal, ml.	Reduction of adrenal ascorbic acid, left gland–right gland (L–R), mg./100 gm. fresh adrenal tissue	
			Individual values	Mean
1 Woman 32	Cushing's syndrome. Adrenal cortical hyperplasia	2.0–3.0	31.1, 22.5	26.8
2 Woman 36	Cushing's syndrome. Adrenal cortical hyperplasia	2.1	10.7, −17.1	− 3.2
3 Woman 24	Cushing's syndrome. Adrenal cortical tumor	2.5	56.0, 36.1, − 5.0	29.0
3′	As above. 12 days after operation	2.5	10.6, 12.5, 44.6	22.6*
4 Woman 27	Virilizing adrenal tumor without Cushing's syndrome	2.5	9.1, −26.7, 18.1	0.2
5 Woman 25	Cushing's syndrome, persistent 5 mo. after subtotal resection of one hyperplastic adrenal	1.5	−31.4, −26.4	−28.9
6 Woman 32	Cushing's syndrome, 10 days after subtotal resection of one hyperplastic adrenal	2.5	16.9, −18.6, −28.8	−10.2*

Group mean = 4.8 ± 10.6

* These are not included in the group mean because of recent operative procedure.

either to tumor or to hyperplasia. All of the patients exhibited clinical and laboratory evidence of adrenal cortical hyperfunction at the time the blood was drawn, including those who were studied postoperatively. Two patients with hyperplasia (table 4, nos. 1 and 2), and 2 with tumor (nos. 3 and 4) had not had previous surgical treatment. One patient (no. 5) had persistence of Cushing's syndrome following subtotal resection of one hyperplastic adrenal gland five months previously. Blood from 1 patient (no. 6) with hyperplasia was taken ten days after the subtotal resection of one gland. (Values obtained from this patient, however, were not averaged with the others because of the recent major surgical procedure.) Also, blood was obtained twelve days after the surgical removal of a tumor from one of the patients whose blood was studied preoperatively as well (no. 3′).

No consistent results were obtained from the serums of this group

TABLE 5. ASSAY OF ADRENOTROPHIC ACTIVITY IN THE SERUM OF PATIENTS HAVING ADRENAL CORTICAL INSUFFICIENCY

Patient, sex and age, years	Diagnosis	Serum volume per test animal, ml.	Reduction of adrenal ascorbic acid, left gland-right gland (L−R), mg./100 gm. fresh adrenal tissue	
			Individual values	Mean
Comparable cases of adrenal insufficiency				
1 Man 37	Addison's disease. Untreated	2.5	17.3, −23.8, 18.3	3.9
2 Man 41	Addison's disease. No treatment for 3 months	3.0	44.0, 15.8	29.9
3 Woman 54	Addison's disease. Untreated	2.5	8.7, 54.1, 18.4	27.1
4 Woman 59	Addison's disease. Untreated	2.5	13.3, 49.3, 7.6	23.4
5 Woman 65	Addison's disease. Untreated	2.5	31.8, 8.3, 39.4	26.5
			Group mean = 22.2 ± 5.2	
Miscellaneous cases of adrenal insufficiency				
1 (see above)	Addison's disease. Untreated	3.0*	101.7, 36.8	69.2
6 Man 18	Addison's disease. Untreated	2.5*	44.7, 39.7	42.2
7 Woman 48	Addison's disease. (?) Untreated. Elevated 17-ketosteroid excretion	2.5	20.3, 19.8, 25.3, −30.1, 9.9	9.0
8 Woman 27	Addison's disease. Off adrenal cortical extract 48 hours	2.5	24.8, 1.1, 10.4	12.1
5 (see above)	Addison's disease after treatment for 1 week with DOCA 3 mg., testosterone 20 mg. and NaCl 5 gm., daily	2.5	1.1, −18.8	−8.8

* Represents use of serum from external jugular vein.

of patients. The serum from 1 patient with hyperplasia (no. 1), and 1 patient with tumor (no. 3), both of whom had Cushing's syndrome, caused a significant decrease in the ascorbic acid concentration of the right adrenal, but the other 4 serums gave negative results. It is interesting that the serum of patient no. 3 with cortical tumor had comparable positive values both preoperatively and postoperatively. The mean L–R of this group of patients, excluding nos. 6 and 3′, was 4.8 ± 10.6 mg.

Adrenotrophic Activity in Serum of Patients Having Adrenal Insufficiency.—Eight patients with the diagnosis of Addison's disease were studied. All of their serums gave positive responses of varying magnitude (table 5). With the exception of patient no. 8 who had been without treatment for only 48 hours, all the others were previously untreated or had not received any therapy for a period of several months. The serum obtained from patients nos. 1 to 5 all caused a drop in the ascorbic acid concentrations of the right adrenal of the test animals. For these 5 cases the individual mean L–R varied from 3.9 to 29.9 mg. and the group mean was 22.2 ± 5.2 mg.

Serum from the external jugular vein secured from patients nos. 1 and 6 was found to cause mean decreases of 69.2 and 42.2 mg., respectively. The serum of patient no. 8, who had been without treatment for only 48 hours, had the relatively low mean L–R of 12.1 mg. A similarly low value of 9.0 mg. was obtained from the serum of patient no. 7 who had an unexplained high excretion of 17-ketosteroids despite the other criteria of adrenal insufficiency. It is also interesting that the serum obtained from patient no. 5 one week after adequate replacement therapy gave a mean L–R of -8.8 mg., compared to 26.5 mg. prior to treatment.

COMMENT

Since the ability of adrenotrophin to reduce the ascorbic acid concentration of the adrenals in hypophysectomized rats has been established as a distinct and specific reaction, a similar response following the injection of serum should be diagnostic of the presence of adrenotrophin in that serum. Our over-all results would indicate that adrenotrophic activity is present in the serum of patients with adrenal cortical insufficiency, and not present in the blood of normal persons or in patients with adrenal cortical hyperfunction. This statement, however, must be qualified in several respects.

The first qualification pertains to the small number of cases studied. However, the small number is perhaps pardonable when it is recalled that both untreated hypofunctioning and hyperfunctioning adrenal cortical states are extremely rare disorders. In spite of this drawback, we feel that the results obtained are indicative of a difference in these situations with respect to adrenotrophic content. Thus, serum from normal persons did not differ significantly from the saline controls ($t = 0.34$, $p = 0.7$). Neither did the serum of patients having adrenal cortical hyperfunction appear different, although the variability encountered in hyperfunctioning states makes statistical analysis untenable. In contrast, serum obtained from patients with adrenal insufficiency gave positive responses in all cases, being significantly elevated from the saline controls ($t = 4.4$, $p = <0.01$), and from normal serum ($t = 3.3$, $p = <0.01$). Thus there would appear to be significant amounts of adrenotrophic material in

the serum of patients with primary cortical hypofunction. This is in general agreement with the concept of an inverse relationship between adrenal function and adrenotrophic function of the pituitary, since all of our cases were considered to be primary adrenal insufficiency. This view is further supported by the studies on 1 patient with adrenal insufficiency whose serum contained adrenotrophic activity before treatment, but none after one week of adequate replacement therapy. The probability exists, however, that in adrenal hypofunction due to pituitary failure, adrenotrophic activity in the serum would be low to absent. Such cases, however, have not yet been studied.

The second qualification applies to the limited amount of test serum that we were able to administer to the individual rats. It is to be recalled that approximately 2.5 ml. of serum were used in all tests, since this was found to be the largest volume that could be injected without evidence of general toxicity and death. It is probable that adrenotrophic activity could be detected with a larger amount of serum, concentrated or purified in such a way as to render it nontoxic. Methods for accomplishing such a purification would be at best empiric until whole serum can be successfully assayed. Therefore, our results do not indicate the absence of adrenotrophin in the blood of normal and hyperfunctioning adrenal cortical states, but merely a concentration too low for 2.5 ml. of whole serum to produce a response under the conditions of the test.

The third qualification pertains to the results obtained in hyperfunctioning adrenal cortical states. The wide variability encountered obviates a decision as to the presence of adrenotrophin in these conditions. Some of the values would seem to indicate increased activity, but the mean of the group as a whole is not significantly different from that of normal serum. Here one may be dealing with two entities with respect to adrenotrophic activity. Hyperplasia may or may not represent a primary pituitary disorder with resultant increased adrenotrophic activity, but active cortical tumor probably represents a pure adrenal disease and as such should not be associated with elevated adrenotrophic activity. It is regretted, however, that our cases have been too few to permit us to draw accurate conclusions regarding the adrenotrophic content of the serum in adrenal cortical hyperfunction.

Apart from these qualifications, these experiments indicate that it is possible to detect adrenotrophic activity in the serum of patients having adrenal cortical insufficiency with the present assay method. With this as a basis, it should be possible to improve the method in the following ways: (1) reduction of the variability of response so as to make significant an L–R value of 10 mg. or less, (2) purification of serum so as to permit the administration of large volumes without toxicity, or (3) the use of jugular blood, which may contain a higher concentration of adrenotrophin than peripheral venous blood. With

such improvement, clear-cut levels of adrenotrophic activity in normal as well as pathologic states may be established.

SUMMARY

Adrenotrophic activity has been demonstrated in the blood of patients with adrenal cortical insufficiency. Conclusive demonstration of adrenotrophic activity in the blood of normal subjects and of patients with adrenal cortical hyperfunction was not achieved.

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NOTES AND COMMENTS

THE DIABETOGENIC EFFECT OF DEHYDROASCORBIC ACID

Of the known naturally occurring compounds, dehydroascorbic acid has a structure that most nearly approaches that of alloxan. The physical and chemical properties of these two compounds are strikingly similar. Dehydroascorbic is prepared by the oxidation of ascorbic acid with quinone (Moll and Wieters, 1936). It produces a characteristic excitement in rats with an LD50 equal to 320 mgs. per kilogram (120 gram rats). The surviving rats tolerate a second dose, three to four times as large as the first. The action of dehydroascorbic acid in producing a diabetogenic state is summarized in Table 1. The results are similar to those obtained following the injection of estrogens (Ingle, 1941; Ingle, Nezamis and Prestrud, 1947) and adrenal steroids (Ingle, 1941) in forced-fed rats, except that the effects are more pronounced and there is no need for special diets. A large dose of dehydroascorbic acid produces hyperglycemia lasting several days. Repeat doses after the blood sugar returns to normal again produces hyperglycemia. There is an increasing effect with increasing dose. Smaller doses injected daily produce hyperglycemia. Following a large dose of dehydroascorbic acid, there is the same triphasic hyperglycemia, hypoglycemia and hyperglycemia as is seen following alloxan injection. The possible position of dehydroascorbic acid as a mediating substance in the diabetogenic effects of estrogens and adrenal steroids, and the possible action of alloxan on an ascorbic acid-dehydroascorbic acid system in the beta cells are under further investigation.

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TABLE 1. EFFECT OF DEHYDROASCORBIC ACID ON BLOOD SUGAR

No. rats	Weight		Dose dehydro-ascorbic acid mg.	Average blood sugar mg./100 cc.									
	Range grams	Average grams		0hrs.	3 hrs.	8 hrs.	1 day	2 days	3 days	4 days	5 days	6 days	7 days.
4	116–120	118	40+130	110	120	125	110	125	330	182	156	132	130
2	115–118	117	20+80	144	121	98	229	150	117	118			
3	118–122	120	20+80	112	111	82	96	131	116	99			
5	116–120	119	20+60	138	125	114	123	176	112	116			
6	120–140	128	30	105			131	137	113	131			
5*	110–160	136	60 per day	123			122	201	247	232	173†	134†	141‡

* Same rats as in group 4 having received 60 mg. 8, 12 and 16 days previously, reinjected as shown.
† Four rats.
‡ Two rats.

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EXOPHTHALMOS IN RATS AFTER PROLONGED ADMINISTRATION OF PROPYLTHIOURACIL

Rats of a Wistar strain were kept on diets containing 0.02% propylthiouracil for many months. After about three months it was observed that some of the animals became exophthalmic. By the end of seven months almost every animal receiving propylthiouracil showed exophthalmos. Control rats on similar diets without propylthiouracil did not. The exophthalmos can be seen best when the animals are active and alert and are attentive to their surroundings. It may not be apparent at all when the eyes are closed in sleep or in anaesthesia. All animals receiving the drug are underweight compared with controls and show typical myxedematous changes—scanty coarse hair, rough skin, and low metabolic rate (−30 to −40%).

FIG 1. This rat received a diet containing 0.02 per cent propylthiouracil in the diet for a period of 22 weeks. Note the exophthalmos.

While the exophthalmos is striking, its anatomical basis is not clear. In the animals autopsied up to the present, the weights of the eyeballs and of the orbital contents were not greater than in controls. The quantity of retrobulbar fat was negligible. The lymphocytic infiltration of retrobulbar tissues which has been described in Graves Disease (Salter and Soley 1944) was not found in these animals.

These observations do not entirely preclude the possibility of some edema of the orbital tissues but they do suggest that other factors such as pressure from the muscular floor of the orbit or abnormal retraction of the eyelids are more important.

One is tempted to draw a parallel between these observations and the exophthalmos which has been produced by the injection of thyrotropic hormone into guinea pigs and to the progressive exophthalmos which sometimes follows the treatment of Graves Disease by thiouracil derivatives (Williams and Bissell 1943; Haines and Keating 1946). It is not, however, our intention

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to imply such parallelism unless further studies establish their anatomical or pathological similarity.

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TESTICULAR CHANGES IN THE JUVENILE PIGEON DUE TO PROGESTERONE TREATMENT[1]

Nelson (1936) reported that progestin prevents involution of the testicular tubules in hypophysectomized rats. Selye (1940) observed atrophic changes in the testis of the same species after administration of progesterone. In a later paper, however, Selye and Friedman (1941) showed that progesterone caused only a slight atrophy of the interstitial tissue in mice and rats but the seminiferous epithelium remained undamaged. In mature doves results comparable to those reported by Selye and Friedman (1941) for rats and mice, have been obtained after treatment with progesterone (Lahr and Riddle, 1944). However, the action of this hormone on the testis of juvenile birds has not hitherto been reported.

In view of the theoretical interest in the effects of steroid hormones on the testis, it seemed desirable to contribute the present observations, since progesterone treatment was here found for the first time to produce a stimulating action on the juvenile bird testis.

PROCEDURE

Juvenile Indian Rock pigeons (*Columba livia intermedia* Strickl.) 60 days old were involved in this study. The initial body weight of each bird was taken at the commencement of the experiment and the final weight was noted on the day of autospy. Progesterone was injected intra-muscularly into the breast muscles (0.5 mg. in 0.1 cc. of sesame oil daily) for a period of 30 days. The control birds were injected daily in an identical manner with 0.1 cc. of sesame oil alone for the same period.

All the birds were autopsied on the day following the last injections. The testes were carefully dissected out, weighed to the nearest mg., and finally fixed in alcoholic Bouin's fluid for histological studies. Serial sections of the testes were prepared by the paraffin method and stained with Heidenhain's iron-hematoxylin followed by eosin.

EXPERIMENTAL RESULTS

Gross examinations at autopsy revealed pronounced enlargement of the testis of the experimental males in contrast to that of the control birds. A

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[1] Grateful acknowledgment is made to Messrs. Ciba Pharma Ltd. of Bombay for the generous contribution of progesterone used in this study.

perusal of table 1 will also indicate that the testicular weights of the progesterone recipients were significantly greater as compared to those of the control specimens.

Microscopical examination of the testis of the control birds showed a compact appearance typical of immature testis. The tubular epithelium could be identified as a single layer of cuboidal cells. The lumen of the tubules was obliterated in the majority of the cases. The Leydig cells were sparsely distributed in the interstitium which was made up mostly of connective tissue cells.

Histological examination of the testis of the experimental males dis-

TABLE 1. TESTICULAR WEIGHTS IN CONTROL AND PROGESTERONE TREATED PIGEONS

	Number of birds	Testicular weight		Body weight at autopsy
		Absolute	Relative†	
		Mg.	Per cent	Gm.
Control	11	98.2 ± 1.5*	.0000041	203.2 ± 3.5
Progesterone treated	12	175.5 ± 2.1	.0000086	205.1 ± 3.8

† Organ weight expressed as percentage of body weight.
* Standard error of the mean.

closed a much less compact condition. The seminiferous tubules were enlarged in size and the tubular lumen appeared very conspicuous. Divisional stages were abundant in the tubular epithelium which presented a stratified appearance in contrast to its cuboidal condition in the control birds. The Leydig cells showed a marked predominance over the connective tissue cells in the interstitium. The former exhibited a peculiar tendency to form clusters of 3 to 4 cells.

DISCUSSION

Judging from the testis weights, treatment with progesterone significantly provoked testicular stimulation in the juvenile pigeons. The histological consequences of the enhanced testis weights in the treated birds were reflected in the enlargement of the seminiferous tubules and the marked increase in number of the Leydig cells.

A point of considerable interest was the conversion of the seminiferous epithelium from a cuboidal nature in control males to a stratified shape in the treated ones. If this finding is interpreted as an indicator of possible gametokinetic action of progesterone, then it becomes extremely important, since a substantial evidence is provided in favor of the stimulating action of a hormonally active steroid. Another significant item, and that is the marked increase in number of the Leydig cells in the treated males, should also be reckoned in this connection. This finding, therefore, appears to be at variance with the previous reports of Leydig cell atrophy (Selye, 1940; Selye and Friedman, 1941; Lahr and Riddle, 1944) due to progesterone treatment. Moreover, it provides a noteworthy exception to Selye and Albert's (1942) generalization that all hormonally active steroids injure the Leydig cells.

It has been satisfactorily established that the secretions of the anterior hypophysis must be regarded as the chief factors concerned with the regula-

tion of both the gametogenic and hormonal activities of the testis (*Vide* Turner, 1948). In the light of this knowledge, therefore, it can be assumed that in the present material progesterone treatment influenced the hypophysis to release greater amounts of ICSH and FSH which probably accounts for the increase in number of the Leydig cells as well as for the changes in the seminiferous epithelium.

SUMMARY

Intramuscular injections of progesterone (0.5 mg. daily for a period of 30 days) into 60 day old pigeons elicited significant increase in the testicular weights. Evidence is presented to show that the seminiferous epithelium was converted from a cuboidal state in the control males to a stratified shape in the hormone treated ones. There was also marked increase in number of the Leydig cells in the progesterone recipients. It is suggested that these changes were induced by the hormone through the augmentation of FSH and ICSH output of the anterior hypophysis.

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DECREASE OF THE SPREADING ACTION OF HYALURONIDASE IN PANCREOPRIVE DIABETES

The decreased resistance to infection in diabetes is a complex phenomenon, probably due to the following factors:

a) Propagation of infection.
b) Complement activity, bacteriostasis, phagocytosis, etc.
c) Formation of antibodies.

On this hypothesis, we thought it would be interesting to find out the spreading action of hyaluronidase in experimental diabetes. Volterra and Giuli (1934) found a greater spreading action of hyaluronidase for trypan blue (1:300) in human diabetes.

MATERIAL AND METHODS

We used 12 dogs, both sexes, of medium weight (8–12 kgs.), hyaluronidase (Hydase) prepared by "Wyeth", and India ink "Pelikan, 541, Gunther Wagner" as the substance to be spread. Before pancrectomy, in the normal animal, we explored the spreading of India ink by intradermic injection with the following solutions: A). 1 cc. saline solution 8.5% to which 21 drops India ink were added.—B). 1 mg. hyaluronidase ("Hydase") dissolved in

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1 cc. saline solution 8.5% plus 21 drops India ink. The resulting surface was drawn on transparent cellophane paper and measured afterwards by a planigraph.

RESULTS

From Table 1, it is clearly concluded that removal of the pancreas produces a decrease in the spreading action of hyaluronidase in all the cases

TABLE 1. COMPARATIVE RESULTS OF THE SPREADING ACTION IN NORMAL DOGS THE SAME AFTER PANCRECTOMY, WITHOUT INSULIN CONTROL

Dog No.	Spreading action. MM^2		Differences
	Normal	Pancreoprive diabetes	
853	620	570	− 50
855	2,160	1,240	− 920
856	1,200	660	− 540
857	660	600	− 60
862	2,915	1,325	−1,590
865	1,535	1,250	− 285
866	2,225	750	−1,475
868	2,420	2,003	− 417
899	2,520	1,410	−1,110
900	1,900	1,550	− 350
901	3,020	1,240	−1,780
902	1,640	1,030	− 610

TABLE 2. COMPARATIVE RESULTS OF THE SPREADING ACTION IN NORMAL DOGS AND AFTER PANCRECTOMY. DIABETES CONTROLLED BY INSULIN

Dog No.	Spreading action. MM^2		Differences
	Normal	Controlled Pancreoprive diabetes	
865	1,535	2,740	+1,205
868	2,420	4,650	+2,230
899	2,520	1,560	− 960
900	1,900	2,890	+ 990
901	3,020	2,120	− 900
902	1,640	2,430	+ 790

studied. Results scowed in Table 2 are less definite but very significant. In spite of the fact that there are only six cases, their statistical study clearly indicates a similar correlation.

SUMMARY

The spreading action of hyaluronidase in pancreoprive diabetes and the influence that insulin exerts on such a spreading action is studied. We have found that pancreoprive diabetes has an action which decreases the spreading effect. This fact is opposed to the result obtained by Volterra in human diabetes, in which he found a greater spreading action of hyaluronidase with regard to normal individuals.

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ASSOCIATION NOTICE

ANNOUNCEMENT

The membership roll of the Association is being revised in preparation for the issuance of a new Roster as of December 31, 1949. Copy for this must be in the hands of the printer not later than October 1, 1949.

Return postal cards are being mailed to members requesting the following information:

Name..... Degrees.....
Mailing Address.....
.....
Teaching or Research Position:.....
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.....
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Members are urged to complete and return the card immediately and to keep the Secretary informed at all times of any change in the above.

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